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CALCIUM-ACTIVATED CHLORIDE CHANNEL PROTEINS AND THEIR USE IN ANTI-METASTATIC THERAPY

This application is a Continuation In Part of U.S. patent application 10/055,412 filed October 29, 2001, which is a Divisional of U.S. patent application serial no. 09/193,562 filed on November 17, 1998, now U.S. patent no. 6,309,857, which claims the priority of U.S. Provisional Application serial no. 60/065,922 filed on November 17, 1997, the disclosures of which are incorporated herein by reference.

This invention was made with government support under grants CA 47668 from the National Cancer Institute. The government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to nucleotide sequences encoding a family of mammalian calcium activated chloride channels which may alternatively, or additionally function as adhesion molecules. More particularly, the invention is directed to genes isolated from bovine endothelial cells, human endothelial cells and murine endothelial cells, which encode calcium activated chloride channel molecules and include the lung-endothelial cell adhesion molecules and associated proteins.

BACKGROUND OF THE INVENTION

Calcium Activated Chloride Channels

Ion channels are not only required for normal cellular functions but also play a critical role in numerous diseased states. For example, cystic fibrosis results when ion transport in epithelial cells of individuals is altered due to a genetic defect of the cystic fibrosis transmembrane conductance regulator CFTR; Knowles et al., 1983, J. Clin. Invest.71:1410-1417). Although serious airway pathology is usually the primary cause of mortality in young adults with CF, intestinal epithelial alterations have also been observed. However, the severity of tissue lesions does not correlate with the expression of CFTR in

humans or mice, suggesting the involvement of cell-specific channels in addition to CFTR. Further support for the involvement of other channel protein molecules in CF comes from observations that calcium activated chloride secretion is preserved in respiratory epithelia of CF patients compared to unaffected individuals, but is significantly reduced or absent from CFTR-defective epithelia. These results strongly suggest that an alternative non-CFTR regulated chloride channel activity might account for attenuating CF disease in some tissues. Thus, a need exists for identification, isolation and functional analysis of alternative chloride channels.

Adhesion Molecules

It is apparent that endothelial cell adhesion molecules may have functions in addition to their adhesive functions. For example, integrins have transmembrane signaling capacities which may play a role in the adherence process. However, the primary function of endothelial cell adhesion molecules is adherence to a substrate such as (a) to promote adherence of endothelial cells to basement membrane, (b) to promote vascular arrest and to facilitate extravasation of leukocytes such as during an immune response, and (c) to promote homing of lymphocytes to a particular lymphoid tissue. Other molecules may play a role in controlling adherence of endothelial cells. For example, chloride ion channels are thought to be involved in a signaling cascade when lymphatic endothelial cells begin to adhere to a substrate (Martin et al., 1996, Microvasc. Res. 52:200-9).

There is considerable evidence that metastatic nonlymphoid tumor cells mimic leukocytes in recognizing and adhering to one or more endothelial cell adhesion molecules to migrate in blood vessels, to arrest in vascular areas of organs which may provide the microenvironment conducive for metastatic growth, and to extravasate into surrounding tissues. An example of such an endothelial cell adhesion molecule which promotes adhesion of tumor cells and mediates metastasis is lung-endothelial cell adhesion molecule (Lu-ECAM-1). Lu-ECAM-l is a 90 kilodalton (kDa) integral membrane protein constitutively expressed primarily in endothelial cells of pleural and subpleural microvessels. Both in vitro studies and in vivo studies indicate that Lu-ECAM-1-expressing endothelial cells promote adhesion of certain lung-colonizing tumor cells in a manner that is consistent with the expression level of the adhesion molecule and the metastatic propensity of tumor cells. For example, in an in vitro tumor cell/endothelial cell adhesion assay, highly lung metastatic BlG-F10 melanoma cells bind to lung-matrix-

modulated endothelial cells expressing Lu-ECAM-l in significantly larger numbers than their intermediate or low lung-metastatic counterparts (BlG-L8-F1O and B1GFO, respectively; Zhu et al., 1991, Proc. Nati. Acad. Sd. USA 88:9568-720). Such binding appears to be calcium (Ca^{2+}) dependent. Further, anti-Lu-ECAM-1 monoclonal antibodies significantly inhibit adhesion of B1GFl0 melanoma cells to Lu-ECAM-1 expressing endothelial cells in culture (Zhu et al., 1991, supra) Anti-Lu-ECAM-1 monoclonal antibodies are also efficient in preventing metastatic colonization of the lungs by highly lung-metastatic B1GF1O cells in a standard animal model for metastasis (Zhu et al., 1991, supra). Lu-ECAM-l, affinity purified from detergent extracts of bovine aortic endothelial cells, was used to immunize mice. The immunized mice showed an inhibition of metastatic colonization of the lungs by B1GFl0 melanoma cells, the efficiency of which was dependent upon the anti-Lu-ECAM-l serum titer (Zhu et al., 1992, J. Clin. Invest. 89:1718-1724). Lu-ECAM-l appears to be the endothelial cell adhesion molecule for metastatic tumor cells that express the ligand /34 integrin subunit (and possibly other ligands) including, but not limited to, lung-metastatic breast tumor cells, and lung-metastatic melanoma tumor cells.

Anti-adhesion therapy may be used to interfere with adhesion between organ-specific endothelial cells and blood-borne cancer cells in preventing the formation of metastatic colony formation in organs that support metastatic cell growth. The amount of endothelial cell adhesion molecule that can be made from detergent extracts, as well as the rate of production of the endothelial cell adhesion molecule, is generally insufficient for cost-effective commercial production. More efficient production of proteins, with a concomitant reduction in production cost, can often be achieved by producing a protein through recombinant means. In that regard, in some cases a host cell may be genetically engineered such that an increased amount of the protein is produced and/or the protein is produced in a manner which facilitates its isolation (as compared to harvesting the protein from cell membranes).

SUMMARY OF THE INVENTION

It is an object of the invention to provide nucleotide sequences, isolated from mammalian endothelial cells, which encode molecules that functions as a calcium activated chloride channel-adhesion molecule **(also referred to herein as** "CACC-AM"

or "CLCA").

It is also an object of the present invention to provide nucleotide sequences which are variants (including portions) of the gene comprising the CACC-AM, and which encode a polypeptide having substantially the biological activity as compared to the biological activity of the CACC-AM.

It is an object of the present invention to provide a means for recombinantly producing CACC-AM molecule.

It is an object of the present invention to provide a means for recombinantly producing proteins associated with CACC-AM molecule.

It is a further object of the present invention to provide expression vectors containing a nucleotide sequence that encodes a CACC-AM molecule; or containing a nucleotide sequence which is a variant of the gene for CACC-AM, and that encodes a polypeptide having substantial biological activity of a CACC-AM; or containing a nucleotide sequence that encodes a protein associated with a CACC-AM.

It is an additional object of the present invention to provide recombinant host cells which contain multiple copies of a nucleotide sequence that encodes a CACC-AM molecule, wherein the CACC-AM molecule is recombinantly produced by culturing the recombinant host cells under suitable conditions.

It is an additional object of the present invention to provide compositions and methods for inhibiting metastasis. It is another object of the present invention to provide compositions and methods for inhibiting the growth of metastatic tumors in mammals.

Other objects, features, and advantages of the present invention will become apparent from the following drawings and detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of a method for identifying clones using polymerase chain reaction. Also shown are restriction enzyme sites EcoRI ("R"); NdeI ("N"), PstI ("P"), and BglII ("B").

FIG. 2A is a representation of immunoblots of bovine aortic endothelial cell proteins using either monoclonal antibody D3 ("D3"), polyclonal antibody CUll ("11"), polyclonal antibody CU19("19"), polyclonal antibody R4 ("R4"), and polyclonal antibody R41 ("R41").

FIG. 2B is a representation of Lu-ECAM-l untreated ("-")or Lu-ECAM-l treated with N-glycosidase F ("+") followed by immunoblot analysis using polyclonal antibody R4; and Lu-ECAM-l-associated proteins untreated ("-") or Lu-ECAM-l-associated proteins treated with N-glycosidase F ("+") followed by immunoblot analysis using polyclonal antibody R41.

FIG. 3A is a representation of bovine aortic endothelial cells either untreated ("-") or treated with a crosslinker ("+") followed by immunoblot analysis using either polyclonal antibody R4 ("R4"), or polyclonal antibody R41 ("R41").

FIG. 3B is a representation of bovine aortic endothelial cells which were surface-biotinylated in the absence of ("-") or presence of ("+") a crosslinker followed by detection with streptavidin-horseradish peroxidase.

FIG. 4A is a representation of a ethidium bromide stained agarose gel containing the results of reverse transcriptase polymerase chain reaction analysis of bovine aortic endothelial cells ("BAEC"), lung tissue, tracheal epithelium, and spleen tissue using Lu-ECAM-1 specific primer pairs Ll, and L2.

FIG. 4B is a representation of a ethidium bromide stained agarose gel containing the results of reverse transcriptase polymerase chain reaction analysis of bovine aortic endothelial cells ("BAEC"), lung tissue, tracheal epithelium, and spleen tissue using bovine tracheal chloride channel ("Ca-CC") specific primer pairs T1, and T2.

FIG. 5 is a bar graph illustrating lung-metastatic tumor cell adhesion to wild type Lu-ECAM-1 in the presence or absence of anti-Lu-ECAM-l mAb 6D3; and lung-metastatic tumor cell adhesion to recombinant Lu-ECAM-1 in the presence or absence of anti-LuECAM-l mAb 6D3.

FIG. 6 is a representation of the expression of mCLCA1 by in vitro translation (A) and in transfected HEK293 cells (B)

FIG. 7 is a representation of the biochemical analysis of hCLCA1 protein for in vitro translated (a), c-myc tagged hCLCA1 transfected HEK293 (b), and surface expression of c-myc tagged hCLCAl.

FIG. 8 is a representation of biochemical analysis of the hCLCA2 protein for in vitro translation (a) and immunoblot detection of myc tagged hCLCA2 constructs in HEK293 cells (b)

FIG. 9 is a representation of whole cell currents in mCLCAl transfected HEK293

cells.

FIG. 10 is an illustration of the summary the effects of inhibitors on mCLCA1 current expression.

FIG. 11 is a representation of whole cell currents in hCLCA1-transfected HEK293 cells.

FIG. 12 is an illustration of the summary the effects of inhibitors on hCLCA1 current expression.

FIG. 13 is an illustration of the electrohpysiological analysis of hCLCA2.

FIG. 14 is a representation of a comparison of the amino acid sequences of the calcium activated chloride channels, hCLCA1 (SEQ ID NO:27); hCLCA2 (SEQ ID NO:31); bCLCA1 (SEQ ID NO:46); Lu-ECAM-1 (SEQ ID NO:1); and mCLCA1 (SEQ ID NO:33).

FIG. 15A is a graphical representation of the dissection of the 90-kDa protein of the CLCA prototype bCLCA2 (Lu-ECAM-1) into various GST fusion proteins (GST-HX, -HV, -HP, -NE, -VX, -PX, and -BX).

FIG. 15B is a representation of a Coomassie Blue-stained gel (SDS-PAGE) of GST-bCLCA2 fusion proteins synthesized by *E. coli.*

FIG. 15C is a representation of Western blots testing of the GST-bCLCA2 fusion proteins for binding to the adhesion-blocking anti-bCLCA2 mAb6D3 using immunoprecipitation and Western blotting with anti-GST polyclonal antibodies. *Lane 1*, immunoprecipitated GST-bCLCA2 fusion protein; *lane 2*, starting material: solubilized, purified GST-bCLCA2 fusion proteins. Notice that the shortest bCLCA2 fragment pulled down by mAb6D3 is GST-BX.

FIG. 15D is a graphical representation of results from testing of GST-bCLCA2 fusion proteins (BX, NE, NP, and PX) for adhesion to MDA-MB-231 cells. MDA-MB-231 cells strongly adhere to GST-PX and -BX but do not adhere to GST-NE and -HP. MDA-MB-231 cells also adhere to the 90-kDa and to the 35-kDa subunits of bCLCA2 and hCLCA2.

FIG. 16A depicts a PROTOMAT-identified conserved sequence motif in the 90- and 35-kDa processing products of hCLCA2 is compared with the corresponding sequences in mCLCA5, mCLCA1, and bCLCA1, all expressed by pulmonary endothelia (boxed motifs, principal test sequences; underlined motifs, not tested). Sequences as

shown in Fig. 16A are: hCLCA2 90-kDa β_4BM (AFSRISSGTG), SEQ ID NO:50; hCLCA2 and mCLCA5 have the same 35-kDa β_4BM (GFSRVSSGGS), which is SEQ ID NO:51; the mCLCA5 90-kDa β_4BM (AFVRISSGTG) is SEQ ID NO:54; the mCLCA1 90-kDa β_4BM (AFSRISSTSG) is SEQ ID NO:55; the mCLCA1 35-kDa β_4BM (DFNRVTSGGS) is SEQ ID NO:56; the bCLCA2 90-kDa β_4BM (AFSRISSRSG) is SEQ ID NO:57; and the bCLCA2 35-kDa β_4BM (DFSRLTSGGS) is SEQ ID NO:58.

FIG. 16B is a graphical representation of GST-HA AFSRISSGTG **(SEQ ID NO:50)** (100 ng/ml) representing the β_4-binding motif of the 90-kDa hCLCA2 (termed $\beta_4BM_{hCLCA2(90)}$), which was generated and tested for adhesion to dishes coated with β_4 integrin, β_1 integrin, β_3 integrin, fibronectin, and BSA (10 μg/ml) using anti-GST antibody ELISA to detect bound $\beta_4BM_{hCLCA2(90)}$. Notice that $\beta_4BM_{hCLCA2(90)}$ bound only to β_4-coated dishes, $p < 0.01$ relative to BSA.

FIG. 16C is a representation of Western blotting results from a pull-down (PD) assay of soluble β_4 but not β_1 and β_3 (remain in flow-through (FT)) with $\beta_4BM_{hCLCA2(90)}$ immobilized on glutathione beads. α -β_4 pAbH101, α -β_1 mAb(clone 18), and α -β_3 mAb25E11 were used for Western blot detection.

FIG. 16D is a representation of Western blotting results from a PD of $\beta_4BM_{hCLCA2(90)}$ by immunobead-immobilized β_4 integrin but not by β_1 integrin. α -GST pAb(B-14) was used for Western blot detection.

FIG. 17A is a graphical representation of results from an adhesion assay indicating $\beta_4BM_{hCLCA2(90)}$, but not GST, adheres to β_4 integrin-coated dishes (15 μg/ml) in a dose-dependent manner (assay medium: phosphate-buffered saline + 1 mM $MnCl_2$).

FIG. 17B is a graphical representation of results demonstrating Mn^{2+}, but not Mg^{2+} and Ca^{2+}, promotes adhesion of $\beta_4BM_{hCLCA2(90)}$ (50 ng/ml) to β_4 integrin (15 μg/ml). *, $p <$ 0.01 relative to GST control.

FIG. 18A is a graphical representation of results demonstrating binding of $\beta_4BM_{hCLCA2(90)}$ (100 ng/ml) to MDA-MB-231 analyzed by FACS. Notice that $\beta_4BM_{hCLCA2(90)}$ binds strongly to MDA-MB-231 (*a*) and generates a histogram similar to that recorded for anti-β_4 pAbH101 staining of MDA-MB-231 (*b*); the control polypeptide P14 does not bind.

FIG. 18B is a graphical representation of results demonstrating selective adhesion of MDA-MB-231 cells to $\beta_4BM_{hCLCA2\ (90)}$- and $\beta_4BM_{hCLCA2(35)}$-coated wells (10 μg/ml) (negative control: BSA and GST; positive control: poly-L-lysine (*PLL*)).

FIG. 18C is a graphical representation of results demonstrating inhibition of the MDA-MB-231 adhesion to hCLCA2 by $\beta_4BM_{hCLCA2(90)}$ and $\beta_4BM_{hCLCA2(35)}$ (both 100 ng/ml) but not by the control polypeptides P14 and PEDA. *, $p < 0.01$ relative to MDA-MB-231 adhesion to hCLCA2.

FIG. 19A is a graphical representation of MDA-MB-231 lung colony numbers. MDA-MB-231 breast cancer cells incubated for 20 min with $\beta_4BM_{hCLCA2(90)}$ or GST were injected into the lateral tail vein of 4-week-old, female Scid/beige mice (1×10^5 tumor cells/300 μg of $\beta_4BM_{hCLCA2(90)}$/0.2 ml of Dulbecco's modified Eagle's medium/mouse). A total of eight mice (2 x 4) per test condition were injected. Mice were sacrificed 8 weeks later, and lung colonies were counted and tabulated.

FIG. 19B is a representation of gross and histological views of the lungs of test animals showing multiple tumor colonies in mice injected with GST and normal tumor-free lungs in mice injected with $\beta_4BM_{hCLCA2(90)}$. *T*, tumor.

FIG. 20A is a graphical representation of the CLCA-binding domain's location in the SDL of β_4 integrin. *A,* scheme of the β_4 integrin: *1–27*, signal sequence; *111–343*, putative I domain-like structure; *184–203*, predicted loop region of β_4. Sequences of the loop regions of β_4(184–203) and β_1(197–219) are displayed (*boxed*). The β4 sequence is SEQ ID NO:60; the β1 sequence is SEQ ID NO:62; the E.Coli sequence is SEQ ID NO:63.

FIG. 20B is a graphical representation of results from an hCLCA2 binding assay. Myc-tagged hCLCA2 (3 μg/ml) is bound to uncoated wells (*black column*), GST-(*gray column*), GST-β_1(197–219)-(*dashed column*), or GST-β_4(184–203)-coated wells. Bound hCLCA2 is detected by anti-Myc antibody. hCLCA2 binds to GST-β_4(184–203)-coated wells as well as uncoated wells (positive control) but not to wells coated with GST-β_1(197–219) or GST.

FIG. 20C is a graphical representation of results from a pull-down assay. GST-β_4 and GST-β_1 fusion polypeptides are immobilized on glutathione beads and then tested for

pull-down of hCLCA2 from lysates of transfected HEK-293 cells. Notice that only GST-β_4 is able to pull down hCLCA2.

FIG. 20D is a graphical representation of results demonstrating that GST-β_4 binds to GST-$\beta_4BM_{hCLCA2(90)}$. The wells of microtitration plates were coated with $\beta_4BM_{hCLCA2(90)}$ or control polypeptide PEDA (10 μg/ml each) and tested for binding of biotinylated GST-β_4 (30 μg/ml) by ELISA.

FIG. 20E is a representation of a Western blot demonstrating that chimeric $\beta_{4\text{-}1\text{-}4}$ fails to bind hCLCA2. $\beta_{4\text{-}1\text{-}4}$ was generated as described in Example 11 and transfected together with α_6 into HEK293 cells (positive control: $\beta_4 + \alpha_6$; negative control: α_6). Lysates from transfected HEK293 cells were incubated with anti-β_4 pAbH101-conjugated protein G beads (overnight at 4 °C). Beads were then washed and incubated with immunopurified Myc-tagged hCLCA2. Bound material was detected by Western blotting using anti-Myc mAb 9E10. *WB*, Western blot.

FIGS. 21A-21E are graphical representations of results from adhesion assays demonstrating inhibition of the β_4/CLCA adhesion with a β_4 SDL polypeptide. Wells of microtitration plates were coated with substrate (hCLCA2 (Fig. 21A), mCLCA1 (Fig. 21D), EHS laminin (Fig. 21C and Fig. 21E), or placental laminin (Fig. 21B)) overnight at 4 °C at the indicated concentration, then seeded with MDA-MB-231 (Fig. 21A, Fig. 21B, and Fig. 21C) or 4T1 (Fig. 21D and Fig. 21E) breast cancer cells, and incubated for 20 min at 37 °C. No effect was recorded for the binding to placental and EHS laminins. *, $p <$ 0.01 relative to adhesion to substrate alone.

FIG. 22A is a graphical representation of a CLCA molecule. CLCA molecules (125 kDa) are normally cleaved into 90- and 35-kDa polypeptides. The proteolytic processing site is indicated by an *arrowhead* and marked *PS*. *TM1–5* denote transmembrane domains, and *SS* denotes the signal sequence. Putative β_4 integrin-binding domains in the 90- and the 35-kDa cleavage products are marked β_4BM. The hCLCA2 domain SEQ ID NO:s are as described in Fig. 16A. The hCLCA1 sequences shown are the hCLCA1 90-kDa β_4BM (AFGALSSGNG) which is SEQ ID NO:59 and the hCLCA1 35-kDa β_4BM (CFSRTSSGGS) which is SEQ ID NO:52.

FIG. 22B is a representation of gels showing the purified Myc-tagged hCLCA1 by α -Myc immunoaffinity chromatography. Four fractions (*F1–F4*) were collected from the

column. Fraction 1 and fraction 4 show presence of the 90-kDa subunit only, while fraction 2 and fraction 3 show presence of both the 125- and 90-kDa proteins (α -Myc Western blot).

FIG. 22C 22A is a graphical representation of results demonstrating adhesion of MDA-MB-231 to fractions 1–4 revealed that tumor cells were unable to adhere to fraction 1 and fraction 4 but adhered well to fractions 2 and 3, likely through the preserved β_4BM in the 35-kDa sequence of unprocessed hCLCA1.

FIG. 22D is a graphical representation of results from a pull-down assay demonstrating that the 90-kDa fragment of hCLCA1 and hCLCA2 with glutathione bead-immobilized GST-β_4(184–203). hCLCA2, but not hCLCA1, is pulled down by GST-β_4 (α -Myc Western blot). *PD*, pull-down; *FL*, flow-through.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

"Precursor" is a term used in conjunction with "lung-endothelial cell adhesion molecule" hereinafter for the purposes of the specification and claims to refer to a sequence of amino acids bound to and located upstream from the N-terminal portion of the mature form of a lung-endothelial cell adhesion molecule, wherein the removal of this sequence results in the formation of the "mature form" of the lung-endothelial cell adhesion molecule. A precursor protein is a form of a lung-endothelial cell containing a prepro-region. The prepro-region is made up of amino acids comprising a signal sequence, wherein the signal sequence is cleaved to form the mature form of a lung-endothelial cell adhesion molecule.

"Calcium activated chloride channel - adhesion molecule" or "CACC-AM" **"or CLCA"** is a term used hereinafter for the purposes of the specification and claims to mean a molecule isolated from mammalian endothelial cells that when expressed in cells induces the expression of calcium activated chloride conductance channels.

"Calcium activated chloride channel (s)" is a term used for the purposes of the specification and claims to mean chloride channels whose conductance is activated by calcium as judged by inhibition of conductance by DIDS, DTT or niflumic acid.

"Recombinant calcium activated chloride channel-adhesion molecule" or

"Recombinant CACC-AM" is a term used hereinafter for the purposes of the specification and claims to refer to a CACC-AM molecule produced from a heterologous cell (e.g., other than from vascular endothelial cells), wherein the heterologous cell has been genetically engineered to contain a nucleotide sequence that encodes a CACC-AM molecule.

"Recombinant calcium activated chloride channel-adhesion molecule-associated protein" or "recombinant CACC-AM- associated molecule" is a term used hereinafter for the purposes of the specification and claims to refer to a CACC-AM associated protein produced from a heterologous cell (e.g., other than from vascular endothelial cells) wherein the heterologous cell has been genetically engineered to contain a nucleotide sequence that encodes a CACC-AM associated molecule. "Lung-endothelial cell adhesion molecule-associated protein" is a term used hereinafter for the purposes of the specification and claims to refer to a protein which (a) is smaller in kilodaltons than the mature form of the lung-endothelial cell adhesion molecule, as determined by, for example, sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) or amino acid analysis; (b) is encoded by messages that also encode the lung-endothelial cell adhesion molecule; (c) is antigenically distinct from the lung-endothelial cell adhesion molecule; and (d) is extracellularly associated in a complex (e.g, specific binding) with the lung-endothelial cell adhesion molecule.

By the term "operably linked" is meant, for the purposes of the specification and claims to refer to the chemical fusion (restriction with subsequent ligation) or synthesis of heterologous DNA with a nucleotide sequence that encodes a lung-endothelial cell adhesion molecule or a lung-endothelial cell adhesion molecule-associated protein such that the resultant recombinant DNA molecule is formed in a proper orientation and reading frame for the nucleotide sequence to be transcribed into functional RNA. In the construction of the recombinant DNA molecule, it is generally preferred to position a promoter at a distance upstream from the initial codon of the nucleotide sequence that is approximately the same as the distance in its natural setting (e.g., in an endothelial cell). However, as known in the art, some variation in the distance can be accommodated without loss of promoter function. Likewise, it is generally preferred to position an enhancer element at a distance upstream from the promoter, or incorporated into the promoter sequences as a promoter element, or located between the promoter and the DNA

molecule to be expressed. However, as known in the art, some variation in the placement can be accommodated without loss of the enhancer element's function. "Expression control sequences" is meant, for the purposes of the specification and claims to refer to a promoter or promoter-enhancer combination.

By the term "expression vector" is meant, for the purposes of the specification and claims to refer to a DNA molecule which is operably linked to a nucleotide sequence that encodes one or more recombinant proteins comprising a lung-endothelial cell adhesion molecule and/or a lung-endothelial cell adhesion molecule-associated protein such that the production of the recombinant protein is effected in a suitable host. The vector may include, but is not limited to, a plasmid, phage, or a potential genomic insert.

By the terms "degeneracy substitutions", for the purposes of the specification and claims to refer to the base pair changes (substitutions) in the nucleotide sequence such as a change in one or more bases of a triplet codon (e.g., third base degeneracy) resulting in the encoding of the same amino acid as before the change, or a change resulting in the encoding of a conservative substitution in the amino acid sequence encoded. With respect to such variations, and as appreciated by those skilled in the art, because of third base degeneracy, almost every amino acid can be represented by more than one triplet codon in a coding nucleotide sequence. Thus, in nature or by mutagenic means, the nucleotide sequence be modified slightly in sequence (e.g., substitution of a nucleotide in a triplet codon), and yet still encode its respective gene product of the same amino acid sequence as encoded by the disclosed nucleotide sequences.

Further, the nucleotide sequence may have minor base pair changes which may result in variation (conservative substitution) in the amino acid sequence encoded. Such conservative substitutions are not expected to substantially alter the biological activity of the gene product. A "conservative substitution" for the purpose of the specification and claims means modification of one or more amino acids are such that the tertiary configuration of the recombinant protein is substantially unchanged. Conservative substitutions is defined by aforementioned function, and includes substitutions of amino acids having substantially the same charge, size, hydrophilicity, and/or aromaticity as the amino acid replaced. Such substitutions, known to those of ordinary skill in the art, include glycine-alanine-valine; isoleucine-leucine; tryptophan-tyrosine; aspartic acid-glutamic acid; arginine-lysine; asparagine-glutamine; and serine-threonine. It is noted that a

nucleotide sequence according to the present invention encodes a mammalian Lu-ECAM-1 as to be described more fully herein, and does not encompass the nucleotide sequence encoding the bovine tracheal epithelial chloride channel described recently (Cunningham et al., 1995, J. Biol. Chem. 270:31016-26).

By the terms "% identity of amino acid sequence" are meant, for the purposes of the specification and claims to refer to the percent of amino acid positions that are identical between two amino acid sequences as determined by sequence comparisons performed using algorithms known to those skilled in the art.

By the terms "% identity of nucleotide sequence" are meant, for the purposes of the specification and claims to refer to the percent of nucleotide base pair positions that are identical between two nucleotide sequences as determined by sequence comparisons performed using algorithms known to those skilled in the art.

By the term "substantially" is used in conjunction with the biological activity (e.g., adhesive function or chloride ion channel function) to mean, for the purposes of the specification and claims, to refer to retaining a degree of the biological activity ranging from approximately 50% of the activity to greater than 100% of the activity, in relation to the molecule with which it is compared.

By the term "unexpectedly improved" is used in conjunction with the biological activity (e.g., adhesive function or chloride ion channel function) of a recombinant protein to mean, for the purposes of the specification and claims, to refer to a degree of the biological activity which is approximately greater or equal to 30% more biological activity than that of the molecule to which it is compared, and which improvement in activity was unforeseen for this recombinant protein.

The present invention relates to nucleotide sequences and variants thereof that encode a polypeptide which is a calcium activated chloride channel and/or has adhesion properties. In accordance with this invention, nucleotide sequences encoding Lu-ECAM-l/mouse calcium activated chloride channel (mCLCA), and human calcium activated chloride channel molecules (hCLCA1, hCLCA2, and hCLCA3) are disclosed. The nucleotide sequences have been derived from bovine aortic endothelial cells, from murine aortic endothelial cells, or from human endothelial cells. In one embodiment, a nucleotide sequence of the present invention, SEQ ID NO:l, contains sequences that encode either Lu-ECAM-1 or Lu-ECAM-l-associated protein. From SEQ ID NO:1, the lung-endothelial

cell adhesion molecule precursor is deduced to be approximately 905 amino acids (SEQ ID NO:2). Cleavage of the signal peptide (amino acids -21 to -1 of SEQ ID NO:2) from the lung-endothelial cell adhesion molecule precursor, and subsequent post-translational processing, results in a Lu-ECAM-l of about799 amino acids (amino acid 1 to amino acid 799 of SEQ ID NO:2) and with a predicted molecular size of approximately 87 kDa. It was also discovered during the development of the invention that a SEQ ID NO:1 encodes a Lu-ECAM-l-associated protein (SEQ ID NO:3) which, depending on the glycosylation pattern, has an apparent molecular size (e.g., as determined by SDS-PAGE) ranging from about 22 kDa (little or no glycosylation present) to 38 kDa. More particularly, SEQ ID NO:l encodes Lu-ECAM-1-associated proteins of apparent molecular size of about 38 kDa and of about 32 kDa. Further, these two LU-ECAM-l-associated proteins bind with Lu-ECAM-1 (amino acid 1 to amino acid 799 of SEQ ID NO:2)in forming Lu-ECAM-1 complex. The mCLCA, human CLCA1, and human CLCA2 were then cloned and sequenced using the Lu-ECAM-l open reading frame as a probe.

When compared with the amino acid sequences of previously cloned homologs, hCLCA2 shares a consensus site for monobasic proteolytic cleavage following arginine residue 674 in SEQ ID NO:32. Cleavage at this putative site results in two proteins having amino acids 1-674 and 675-943 of the hCLCA2 full length sequence, which is consistent with the sizes of the 90 kDa and 35 kDa subunits, respectively. (Fuller et al., (2001) Pfluegers Arch. 443, Sippl. 1, S107-S110). A similar putative cleavage site is observed in hCLCA2 at an arginine at position 670 in SEQ ID NO:28 which results in the 90 kDa and 35 kDA hCLCA1 subunits.

In accordance with another embodiment of this invention, using recombinant techniques a nucleic acid molecule containing the nucleotide sequence encoding calcium activated chloride channel-adhesion molecule is incorporated into an expression vector. The recombinant vector is introduced into an appropriate host cell thereby directing the expression of the sequence in that particular host cell. The expression system, comprising the recombinant vector introduced into the host cell, can be used to produce recombinant CACC-AM, or associated proteins. According to the present invention, recombinant CACC-AM, a recombinant polypeptide having CACC-AM activity, and/or recombinant CACC-AM associated protein, can be purified by methods known in the art including ion-exchange chromatography, affinity chromatography, or other chromatographic separation

techniques.

Another embodiment of the present invention is a method for providing calcium-activated chloride conductance channels to mammalian cells. In mammalian cells in which the membrane chloride ion channels are deficient in number or function (e.g., in airway epithelial cells of cystic fibrosis patients), a method of providing to mammalian cells a calcium-activated chloride conductance channel, comprising CACC-AM or a polypeptide having CACC-AM activity, comprises administering directly to the cells an expression vector. The expression vector contains a nucleic acid molecule operably linked to expression control sequences, wherein the nucleic acid molecule encodes a CACC-AM, with the resultant expression vector being introduced into the mammalian cell, and the calcium-dependent chloride conductance produced in the mammalian cells containing the expression vector.

The bovine Lu-ECAM-1 complex appears to be expressed in lung, spleen, and aortic epithelial cells. The murine Lu-ECAM-1 complex appears to be expressed in lung, trachea, spleen, mammary gland, intestine, uterus, epididymis, testis, pancreas, kidney, liver and skin. A first human CLCA1 (hCLCA1) molecule (SEQ ID NO:28) appears to be expressed in small intestine, and colon mucosa. A second human CLCA2 (hCLCA2) molecule (SEQ ID NO:32) appears to be expressed in trachea and mammary gland. A third human CLCA3 (hCLCA3) molecule (SEQ ID NO:30) appears to be expressed in small intestine, trachea, mammary gland, stomach, bone marrow, spleen, lymph node, and peripheral blood leukocytes. That these various mammalian proteins appear to be expressed in tissues which are affected in cystic fibrosis may allow them to be used as chloride channels in accordance with Example 8 herein.

In another embodiment of the invention, a CLCA β4 binding consensus sequence (SEQ ID NO:61) is disclosed. The CLCA β4 binding consensus sequence consists of six amino acids in the order Phe(Ser/Asn)Arg(Ile/Leu/Val)(Ser/Thr)Ser. Peptides comprising the consensus sequence bind to β4 integrin and inhibit adhesion and tumor colony formation of metastatic cancer cells. Further, the β4 binding domains of the 90 kDa hCLCA2 (SEQ ID NO:50), the 35 kDa hCLCA2 (SEQ ID NO:51) and the hCLCA1 35 kDa (SEQ ID NO:52) are also disclosed. Like the CLCA β4 binding consensus sequence,

the hCLCA2 90 kDa, 35 kDa and hCLCA1 35 kDa β4 binding sequences inhibit adhesion and tumor colony formation by metastatic cancer cells.

In yet another embodiment, the present invention provides a method of inhibiting the formation of tumors in an individual. The method comprises the step of administering to an individual a polypeptide comprising the CLCA β4 binding consensus sequence (SEQ ID NO:61). For example, the method of the present invention is useful for administrating the compositions of the present invention to individuals including, but not limited to, individuals who are at risk of developing lung cancer or who have been diagnosed with lung cancer. Methods of administering polypeptides to an individual are well known to those skilled in the art, as are pharmacologically acceptable excipients with which the polypeptides may be combined.

For purposes of the description, the following embodiments illustrate the manner and process of making and using the invention and set forth the best mode contemplated by the inventor for carrying out the invention, but are not to be construed as limiting.

EXAMPLE 1

This embodiment illustrates the molecular cloning of calcium activated chloride channel-adhesion molecules. Lu-ECAM-1. A nucleic acid molecule encoding Lu-ECAM-1 and Lu-ECAM-1-associated proteins according to the present invention can be obtained by preparing cDNA from total RNA isolated from a host cell expressing Lu-ECAM-1. To illustrate this example, total RNA was isolated from bovine aortic endothelial cells by the guanidinium chloride procedure, and a Lu-ECAM-1 CDNA clone was constructed using nucleic acid amplification as summarized in FIG. 1. First, the N-terminal and internal amino acid sequences of a 38 kDa Lu-ECAM-1-associated protein (SEQ ID NO:3) were used to design degenerate primers for primary and nested polymerase chain reactions using the reverse-transcribed total RNA as template. Upstream primers corresponded to nucleotide sequences encoding amino acids 685 to 693, and amino acids 698 to 705, of SEQ ID NO:3. Downstream antisense primers corresponded to nucleotide sequences encoding amino acids 839 to 832, and amino acids 852 to 846, of SEQ ID NO:3. A product of approximately 450 bp was amplified (illustrated in FIG. 1 as "P1"). From these sequences, nondegenerate primers (SEQ ID

NOs: 4 and 5) were designed, and the resultant amplification for 3' sequences resulted in a product of approximately 750 bp (FIG. 1, "P2"). Nondegenerate primers (SEQ ID NOs: 6 and 7) were designed, and the resultant amplification for 5' sequences resulted in a product of approximately 1000 bp (FIG. 1, "P3"). To obtain the remaining 5' sequences (FIG. 1, "24") including a signal sequence and the ATG initiation codon, used was an internal primer (SEQ ID NO:8). To reconstitute the CDNA sequence from the amplified products (Pi-P4), the overlapping products were assembled into one open reading frame by an over-lap extension strategy using a high fidelity polymerase combination. The result was clone 1 (FIG. 1) comprising 3.3 kb and encoding the amino acid sequence of SEQ ID NO:2. Hydrophilicity analysis revealed six significant generally nonpolar regions. In particular, a hydrophobic sequence from amino acid 595 to amino acid 618 appears to be a transmembrane domain. Nine potential sites exist for asparagine-linked glycosylation.

Using the primers to probe a lambda cDNA library, three additional clones (clones 2, 3, and 4; FIG. 1) were identified and sequenced. Additional primers (SEQ ID NOs: 9 and 10) were used to obtain the 5' end sequences. Clone 2, a 3.3 kb variant of clone 1, was identical to clone 1 from nucleotide 252 to nucleotide 2438 of SEQ ID NO:1, but then the sequence diverged. The amino acid sequence deduced from clone 2 (SEQ ID NO:11) was identical to that of clone 1 up to amino acid 772 (of SEQ ID NO:2) followed by a glutamate and serine. Clone 3 was 2.8 kb variant of clone 1. The amino acid sequence deduced from clone 3 (SEQ ID NO:12) was identical to that of clone 1 up to amino acid 772 (of SEQ ID NO:2), followed by an additional 28 amino acids. Clone 4, of 1.3 kb, appears to encode a truncated 321 amino acid (SEQ ID NO:13) variant of Lu-ECAM-1 that may be secreted, and is identical in sequence to amino acids 1 to 303 of SEQ ID NO:2, followed by 18 divergent amino acids. An oligonucleotide probe (SEQ ID NO:14) synthesized from the unique 3' region of clone 1 was used to hybridize MRNA isolated from bovine aortic endothelial cells. The probe detected high molecular weight bands (6-10 kb) in Northern blot analysis as well as the 3.3 kb band. However, the probe did not hybridize to the 2.8 and 1.3 kb bands. These results indicate that the 38 kDa and 32 kDa proteins appear to be encoded only by the messages that also encode the 90 kDa protein.

This embodiment also illustrates that CACC-AM is conserved in mammalian

species, and thus may serve the same or similar functions in mammalian species other than the ones disclosed herein. Conservation of the gene encoding CACC-AM was determined by multispecies genomic DNA (from human, green monkey, rat, mouse, dog, bovine, rabbit, chicken, and budding yeast) blot with probes derived from various regions of the bovine cDNA sequence for Lu-ECAM-1. These probes hybridized to all mammalian species genomic DNA, although the hybridization to rat DNA was comparatively weak. No hybridization signal was detected for chicken DNA or yeast DNA. These results indicate that the gene(or variant sequence thereof) encoding Lu-ECAM-1 is highly conserved in mammalian evolution.

Accordingly, using similar methods and primer sequences for isolating and sequencing of a nucleotide sequence encoding a bovine Lu-ECAM-l, various nucleotide sequences encoding other CACC-AMs maybe identified.

Mouse Calcium Activated Chloride Channel

As an illustration, a murine CACC/AM has been identified. A mouse lung cDNA library in lambda-gtll was screened with the open reading frame of Lu-ECAM-1 cDNA (EcoRl-BglII 2.4 kb fragment of the Lu-ECAM-1 cDNA) using low stringency hybridization conditions (hybridization at 65 C in 5 x SSC, 5x Denhardt's solution and 0.2% SDS solution overnight with agitation; washing with 2 x SSC followed by several washes in 0.2 x SSC, 0.2% SDS at room temperature for a total of 30 minutes). Positive phages were purified and analyzed by Southern blot hybridization techniques. Standard sequencing techniques (eg. automatic sequencing techniques) were used to determine the sequence of the clones. The largest of the isolated cDNA was 2.2 kb in length. It lacked the 5' end as determined by sequence comparison with the known bovine homolog. A full length mouse Lu-ECAM-l was constructed by amplification of the 5' cDNA ends from a pool of mouse lung poly(A)+ RNA (CLONTECH). A gene-specific primer (SEQ ID NO:35) was used to reverse transcribe the cDNA from mouse lung mRNA. A nested primer (SEQ ID NO:36) and a primer recognizing the 5'terminal tag were used to amplify the 5'end of the cDNA by polymerase chain reaction. PCR products were cloned into an expression vector(pGEM-3; Promega). A full length mouse mCLCAl was assembled by fusing the rapid amplification product clone with the 2.2 cDNA insert in an expression vector (pmlI site of pBluescript, Stratagene). Thus a 3.02 kb long sequence (SEQ ID NO:33) encoding a polypeptide of 902 amino acids

(SEQ ID NO:34) was obtained.

Human CLCA1

In another illustration, a nucleic acid molecule encoding human calcium sensitive chloride channels was obtained from either the genomic library or a cDNA library. A human genomic library was screened with the ORE of bovine Lu-ECAM-1 as probe using standard plaque hybridization techniques. Three positive clones of 4,6, and 7 kb were isolated and sequenced, spanning a contiguous genomic fragment of 14 kb with interspersed segments of 58 to 65% nucleotide identity to parts of the Lu-ECAM-1 ORE. Since the regions of homology did not encode a contiguous open reading frame and did not cover the entire Lu-ECAM-1 ORF the remaining parts of the gene were obtained by genomic walking using nested PCR primers from each 5' and 3' end of the clones obtained by plaque hybridizations. Nested PCR conditions were 20 cycles for the first amplification step and 30 cycles for the second amplification with annealing temperatures of approximately 2° below the calculated melting point of the primers and extension times of 5 mm per cycle. PCR products were cloned into a vector (pGem-T, Promega) and sequenced. The full length gene was isolated and sequenced spanning 31,902 bp. The reading frame of the genomic sequence was determined according to its sequence homology with bCLCA1, Lu-ECAM-1 and mCLCA1.

Using an RT-PCR based strategy, the CLCA1 cDNA was cloned and sequenced from small intestinal mRiNA. PCR primers (downstream primer SEQ ID NO:37, and upstream primer SEQ ID NO:38) flanking the ORE and containing linkers with NotI restriction sites were generated and used to amplify the 2745 bp ORF. RT-PCR was performed with 500 ng of human small intestinal poly(A+) (CLONTECH). Reverse transcription was carried out at 48°C with Superscript RNase H- reverse transcriptase and PCR was performed with Pwo DNA polymerase (Boehringer). PCR conditions were as follows: initial denaturation at 94°C for 3 min followed by addition of DNA polymerase; 35 cycles of 94°C for 50 s, 58°C for 30 s, and 72°C for 2 min with a time increment of 3 s per cycle for each extension step, followed by a final extension step of 72°C for 8 min. Foe obtaining the untranslated region of CLCA1 mRNA, amplification of the 5' and 3' ends was carried out using primers SEQ ID NO:39 and SEQ ID NO:40 respectively. The resulting cDNA sequence (SEQ ID NO:27) comprises 3007 bp and is identical to the genomic fragments with high sequence similarity to the previously

cloned homolog. It contains a single ORF of 2745 bp encoding a polypeptide of 914 amino acids (SEQ ID NO:28).

hCLCA2 cDNA

A human lung cDNA library (Clontech) was screened using Lu-ECAM-1 cDNA as probe as described above. Missing 5' and 3' ends of the isolated cDNA species were completed using RACE (Life Technologies). A single 3.6 kb cDNA species was identified and termed CLCA2. A sequence of 2970 bp is shown in SEQ ID NO: 31. The open reading frame of The nucleotide sequence encoding a polypeptide of 943 amino acids (SEQ ID NO:32) shared high degrees of identity with those of Lu-ECAM-1 (86%), bCLCA1 (85%), mCLCAl (76%), and hCLCAI (63%) - Figure 14.

hCLCA3 cDNA

A human spleen cDNA library packed in phage λgtll (Clontech)was screened using standard plaque hybridization protocols. The open reading frame (ORF) of the Lu-ECAM-1 cDNA was used as probe as described above. Phage colony blots were hybridized and washed at low stringency conditions (hybridization: 55°C overnight in 4xSSC standard hybridization buffer without formamide; two stringency washes with 2xSSC, 0.1% SDS at room temperature, and two washes with lxSSC, 0.1% SDS at 40°C). After exhaustive screening of the library (>7 x 10^6 plaques), a single positive phage clone was plaque-purified, amplified, and subjected to DNA purification (Wizard Lambda Preps, Promega). The insert was cut out using the EcoRI sites and cloned into pBluescript II SK (Stratagene). Automated sequencing with initial plasmid-derived primers followed by internal gene-specific primers was performed by the Cornell University DNA Sequencing Facility using dRhodamine Terminator Cycle Sequencing on an ABI Prism 377 DNA Sequencer (PE Applied Biosystems). Missing 5' and 3' ends of the cDNA were isolated using the rapid amplification of cDNA ends (RACE) technique (Life Technologies) and human spleen poly-A+RNA (Clontech) as template. The primers for amplification of 5' end were SEQ ID NO:43 and SEQ ID NO:44, and the primers for 3' end was SEQ ID NO:45. The resulting cDNA sequence of 3599 base paris (deposited in GenBank under accession no. AF043976) was obtained. A sequence of 3418 bp is shown in SEQ ID NO:29, which encodes for a polypeptide of 1000 amino acids (SEQ ID NO:30).

EXAMPLE 2

This example illustrates the proteins encoded by the cDNAs isolated in Example 1 and the relationship between CACC-AM and associated proteins. As an illustration, the relationship is between Lu-ECAM-l and Lu-ECAM-l associated protein is demonstrated. Antigenic characterization was performed by generating anti-Lu-ECAM-l antibodies, and testing the antibodies in Western blot analyses of bovine aortic endothelial cell extracts. Rats were immunized with either the 90 kDa band excised from a polyacrylamide gel and mixed with adjuvant, resulting in polyclonal antibody R4; or a 38 kDa band excised from a polyacrylamide gel and mixed with adjuvant, resulting in polyclonal antibody R41. Two peptides (SEQ ID NOs: 15 and 16) were synthesized, conjugated to KLH, and used to immunize rabbits in forming polyclonal antibodies CUll and CU8, respectively. Monoclonal antibody 6D3 has binding specificity to Lu-ECAM-1 as described previously (Zhu et al., 1992, supra).

As shown in FIG. 2A, mAb 6D3 detected a 90 kDa component (Lu-ECAM-1) and two larger bands of approximately 120 kDa and 130 kDa (Lu-ECAM-1 precursors); but not the 38 kDa or the 32 kDa components (Lu-ECAM-l-associated proteins). Likewise, polyclonal antibody (against amino acid residues of SEQ ID NO:15)recognized only the 90 kDa, 120 kDa, and 130 kDa components(FIG. 2A). In contrast, polyclonal antibody CU19 (against amino acid residues 618 to 767 of SEQ ID NO:2) strongly detected the 38 kDa and 32 kDa components, and the 120 kDa and 130 kDa components, but only weakly detected the 90 kDa component. These results are evidence that the initial translation products of the open reading frame in SEQ ID NO:l are the 120 kDa and 130 kDa components, which are then processed to yield the 90 kDa, 38 kDa, and 32 kDa components.

These results were confirmed with polyclonal antibodies R4 and R41. R4, a polyclonal anti-90 kDa protein antibody, detected the 90 kDa band, as well as the 120 kDa and 130 kDa components; but not the 38 kDa, and 32 kDa components (FIG. 2A). R41, a polyclonal anti-38 kDa protein antibody, detected the 38 kDa and 32 kDa bands, as well as the 120 kDa and 130 kDa components; but not the 90 kDa component (FIG. 2A). These results indicate that (a) the 38 kDa and 32 kDa bands are antigenically related; (b) the 120 kDa and 130 kDa bands are antigenically related; and (c) the 120

kDa and 130 kDa bands have sequence in common with both the 90 kDa protein, and the 38 kDa and 32 kDa proteins. Treatment of Lu-ECAM-l complex with Nglycosidase F reduced the 38 kDa and 32 kDa components to a common band of about 22 kDa, indicating the these two proteins represent alternate glycoforms (FIG. 23). N-glycosidase F treatment reduced the 90 kDa protein to 77 kDa (FIG. 23). The 77 kDa and 22 kDa products would add up to the exact size of the initial translation product of clone 1 before processing.

As shown in FIG. 2A, the 38 kpa and the 32 kDa components of the Lu-ECAM-l complex are not recognized by mAb 6D3 in SDS-PAGE and Western blot analysis, suggesting that these components are likely noncovalently complexed with the 90 kDa protein. The Lu-ECAM-l complex is resistant to dissociation by high salt, detergent, and EDTA, but readily dissociates when boiled in SDS in the presence or absence of reducing agents (e.g., dithiothrietol). To visualize the Lu-ECAM-l complex, and to determine whether the proteins of the complex are associated intracellulary or extracellularly, the surface of bovine aortic endothelial cells was cross-linked. Confluent bovine aortic endothelial cells were surface biotinylated in the presence or absence of disuccinimidyl tartarate (DST), a reagent that restricts cross-linking to extracellular moieties of proteins in close contact. DST dissolved in dimethyl sulf oxide was added to the cells in a final concentration of 1mM. Cross-linking was carried out at 4°C with gentle shaking. The reactions were stopped by adding glycine to a final concentration of 50mM. After quenching for 5 minutes, the cells were lysed for 1 hour in lysis buffer. Lysates were clarified by centrifugation, precipitated with mouse-IgG agarose beads, then immunoprecipitated with mAb 6D3. Immunoprecipitated proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, and detected using avidin-horseradish peroxidase and chemiluminescence. As shown in FIG. 3A, immunoblots using either R4 (polyclonal anti-90 kDa protein antibody) or R41 (polyclonal anti-38 kDa protein antibody) detected a novel band migrating at approximately 140 kDa (arrow, FIG. 3A), with a concomitant reduction in intensities of the 90 kDa, 38 kDa, and 32 kDa components. As illustrated in FIG. 32, all Lu-ECAM-1 complex components were biotinylated on bovine aortic endothelial cell surface. These results suggest that the Lu-ECAM complex is made up of either the 90 kDa and 38 kDa proteins complexed in an extracellular association, and/or the 90 kDa and 32 kDa proteins complexed in an

extracellular association.

In another illustration of this embodiment, the mCLCAl protein was characterized. An in vitro transcription and translation system (TNT™, Promega) was used for the in vitro expression of the full length cDNA (SEQ ID NO:33). Canine microsomes were used to glycosylate the product of in vitro translation. In addition, HEK293 cells were transfected with the cDNA of mCLCA1 using standard methods known to those skilled in the art ($CaPO_4$ or Lipfectamine, Life Technologies). Products were analyzed on SDS-PAGE gels. In addition, mCLCA1 cDNA was also used for transfection of cells. Proteins prepared by standard in vitro translation techniques or from lysates of transfected HEK293 cells were analyzed on Western blotting by using rabbit polyclonal antibodies against N-terminal (CU8) and the C-terminal region (CU2l) of Lu-ECAM peptide. As shown in Figure 6, protein bands of 130, 125, 90 kDa and triplet bands of 32-38 kDa were detected in transfected cells. CU8 reacted exclusively with the large sized bands of 90, 125 and 130 kDa whereas CU21 reacted with only the triplet of the smaller bands. This recognition pattern is similar to that observed for Lu-ECAM-1 and suggests that the ORF of mCLCA1 cDNA encodes a precursor protein, represented by alternate glycoforms of 125 and 130 kDa, that is posttranslationally processed into 90 kDa and 38/32 kDa components.

In another illustration of this embodiment, the hCLCA1 protein was characterized. The ORF of the hCLCA1 cDNA encodes a 914 amino acid protein with a calculated molecular weight of 100.9 kDA. In vitro translation of human CLCA1 cDNA yielded a single protein of approximately 100 kDa, consistent with its calculated size (Figure 7). In the presence of canine microsomes the Mr of the polypeptide shifted to 125,000 indicating multiple glycosylations. Similar to Lu-ECAM-1 and mCLCA1, 37-40 kDa proteins were not detected in immunoblots of whole cell lysates but were coimmunoprecipitated with the 90 and 125 kDa protein. To ascertain whether the 125 kDa hCLCA1 protein is processed into 90 kDa and 30-40 kDa cleavage products in a manner similar to Lu-ECAM-l, c-myc tags were inserted in five different hydrophilic sites with high surface probability (m1-m5) and were overexpressed in HEK293 cells (Cravchik et al., 1993, Gene 137:139-143). Immonoblots of whole cell lysates probed with anti-myc antibodies revealed proteins of 125 and 90 kDa (Figure 7b). However, immunoprecipitation of cell lysates following surface biotinylation indicated the

presence of 37-41 kDa proteins similar to Lu-ECAM-l and mCLCAl (Figure 7c).

In another illustration of this embodiment, the human CLCA2protein was analyzed. The predicted size of the full length protein (104 kDa) is consistent with the result of an in vitro translation assay yielding primary translation product of approximately 105 kDa (Figure 8a) To ascertain whether the CLCA2 protein is cleaved into two subunits in mammalian cells as reported for other CLCAs, two constructs were generated with a c-myc tag within the amino or carboxy terminus respectively as described by Cravchik et al., 1993, Gene 137:139-143) and transfected into HEK293 cells. Immunoblots of cell lysates probed with anti-myc antibody identified an 86 kDa protein when the tag was inserted near the amino terminus (ml) and a 34 kDa protein when the tag was inserted near the amino terminus (m2)-Figure 8b.

EXAMPLE 3

Tissue Distribution

This example illustrates the tissue distribution of CACC-AM. As an illustration, the distribution of Lu-ECAM-1/Lu-ECAM-1 complex in the respiratory tree, as demonstrated by immunohistochemistry. Tissue sections were probed with anti- Lu-ECAM-l antibodies. Formalin-fixed sections of bovine trachea were first denatured by boiling for ten minutes in 4M urea in a microwave oven, then probed with polyclonal antibody R4 (raised against denatured Lu-ECAM-1). The sections were then incubated with donkey anti-rat IgG and avidin-peroxidase conjugate. The peroxidase conjugate was detected using diamino-benzidine as substrate, and then the slides were counterstained with hematoxylin. Lung sections were prepared and probed with mAb 6D3 as previously described (Zhu et al., 1993, mt. J. Cancer 53:68-633) except that a biotinylated secondary antibody was used, followed by the avidin-peroxidase conjugate, diamino-benzidine as substrate, and counterstaining with hematoxylin. The immunohistochemical analyses revealed that Lu-ECAM-l/Lu-ECAM-1 complex was expressed predominantly in endothelia of small to medium-size venules of the lung, and in the respiratory epithelia of bronchi and trachea. To confirm the distribution of expression of Lu-ECAM-l/Lu-ECAM-l complex, and to distinguish it from that of the bovine epithelial chloride channel ("Ca-CC") described recently (Cunningham et al.,

1995, supra), nucleic acid amplification was performed using specific primers as described herein in Example 4.

Tissue distribution for other CACC-AMs of the present invention were determined by Northern blot analysis and RT-PCR. Human multiple tissue Northern blots (Clontech) contained 2 μg poly-A+ RNA per lane of the following tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon mucosa, peripheral blood leukocytes, stomach, thyroid, spinal cord, lymph node, trachea, adrenal gland, and bone marrow. Blots were hybridized labeled fragments for respective cDNAs. To exclude cross hybridization of related family members, highly stringent washing conditions were employed following the hybridization (two washes with 2xSSC, 0.1% SDS at 65°C for 30 min, followed by two washes with 0.2xSSC, 0.1% SDS at 65°C for 30 min). RT-PCR was performed using the above-mentioned conditions and primers to detect the cDNA fragments in poly-A+ RNA samples from human tissues. PCR products were analyzed on an ethidium bromide stained agarose gel. To exclude amplification of a closely related family member, the PCR products were cut out of the gel, cloned into the pGem-T vector, and partially sequenced. In all RT-PCR assays, negative controls were included with water instead of RNA as template in the reverse transcription. To control for RNA quality as well as reverse transcription and PCR conditions, a fragment of EF-la mRNA was amplified as described.

A mouse multiple tissue Northern blot when probed with HindIII fragment of mCLCAl ORF revealed the presence of a 3.1 kb transcript in brain and spleen and transcripts of 5 kb and 3.1 kb in heart, lung, liver, and kidney.

For human CLCA1, a single mRNA species of 3.3 kb was detected in Northern blot hybridizations in small intestine and colon mucosa. Similar results were obtained with RT-PCR.

hCLCA2 mRNA was detected in trachea and mammary gland using the 2832 ORF of hCLCA1. While CLCA2 was not detected in the lung by Northern blot hybridization, the more sensitive RT- PCR revealed its expression in lung in addition to trachea and mammary gland suggesting a significantly lower expression level in the lung.

No signals were detected in any of the tissues tested on Northern blots using the

2817 cDNA of hCLCA3. However, by RT-PCR a fragment of the hCLCA3 cDNA could be amplified form all tissues tested, i.e. spleen, lung, trachea, thymus and mammary gland.

EXAMPLE 4

This example demonstrates that Lu-ECAM-1 and the bovine epithelial chloride channel ("Ca-CC") described recently by (Cunningham et al., 1995, J. Biol. Chem. 270:31016-31026) aredistinct molecules.

1. Genetic similarity

Sequence alignment of the open reading frame of SEQ ID NO:1 with the CA-CC cDNA shows that the nucleotide sequences share 92% identity at the DNA level. Comparing the deduced amino acid sequence of Lu-ECAM-l (SEQ ID NO:2) with that of CA-CC shows 88% identity at the amino acid level. However, the differences appear randomly distributed, and thus, Lu-ECAM-l and CA-CC appear to represent products of different genes.

2. Subunit differences

As shown in FIGS. 2A, 2B, 3A, and 3B, it is clear that the precursor Lu-ECAM-l is a protein with an apparent molecular size of either 120 kDa or 130 kDa. The precursor Lu-ECAM-1 gets processed to a 90 kDa Lu-ECAM-l protein, and to either a 38 kDa or 32 kDa Lu-ECAM-1-associated protein. In contrast, CA-CC is a 140 kDa multimeric complex that can be reduced to a band comprised of 38 kDa subunits in the presence of a reducing agent (Cunningham et al., 1995, supra). This difference in subunit structure is further evidence that Lu-ECAM-1/Lu-ECAM-1 complex is a glycoprotein distinct from CA-CC.

3. Molecular expression differences

It is possible that immunohistochemical staining with polyclonal antibody to Lu-ECAM-1 could detect CA-CC if CA-CC shared a cross-reactive epitope with Lu-ECAM-l. To distinguish Lu-ECAM-1 expression from CA-CC expression in tissues, reverse transcriptase polymerase chain reaction was performed. Messenger RNA (500 ng) from bovine lung tissue, from bovine spleen tissue, from bovine tracheal epithelium, and from cultured bovine aortic endothelial cells was reverse-transcribed with random

oligonucleotide primers and reverse transcriptase in a 20 μil reaction volume. Primers specific for Lu-ECAM-l sequences (primer pairs "Ll":SEQ ID NOS: 17 and 18, "L2": SEQ ID NOs: 19 and 20) , and primers specific for CA-CC sequences (primer pairs "Tl":SEQ ID NOs: 21 and 22, and "T2" SEQ ID NOs: 23 and 24) were confirmed for selectivity by control experiments with a Lu-ECAM-1 CDNA clone. Amplification was performed using 1 μl of the respective cDNA substrate for 35 cycles of amplification in a reaction volume of 50 [Li using 0.5 units of thermostable DNA polymerase, 200 ,uM of each dNTP, 1,5 mM $MgCl_2$, and 1 μM of the respective primer pair. The cycling protocol was 94°C for 20 seconds, 55°C for 10 seconds, and 72°C for 10 seconds, with a time increment of 2 seconds per cycle for annealing and °for 10 minutes. Aliquots (5 μl) of each amplification reaction was fractionated on a 1.5% agarose gel, and stained with ethidium bromide.

The calculated size for product amplified using primer pair L1 is 232 bp; the calculated size for product amplified using primer pair L2 is 218 bp; the calculated size for product amplified using primer pair T1 is 231 bp; and the calculated size for product amplified using primer pair T2 is 218 bp. As shown in FIG. 4A, Lu-ECAM-l is expressed in bovine aortic endothelial cells, lung tissue, and spleen, tissue, but not in tracheal epithelium. In contrast, as shown in FIG. 4B, CA-CC is expressed in lung tissue and tracheal epithelium, but not in bovine aortic endothelial cells nor spleen tissue. These results further support that Lu-ECAM-1 and CA-CC are different molecular entities, with Lu-ECAM-1 being expressed in venular endothelial cells, and CA-CC being expressed in tracheal and bronchial epithelial cells.

EXAMPLE 5

This embodiment illustrates that a nucleic acid molecule comprising a nucleotide sequence encoding CACC-AM, or a variant sequence thereof, or encoding one or more CACC-AM associated proteins, can be inserted into various vectors including phage vectors and plasmids. Successful expression of the protein(s) requires that either the insert comprising the nucleotide sequence, or the vector itself, contain the necessary elements for transcription and translation (expression control elements) which is compatible with, and recognized by the particular host system used for expression. A

variety of host systems may be utilized to express the recombinant protein(s), which include, but are not limited to bacteria transformed with a bacteriophage vector, plasmid vector, or cosmid DNA; yeast containing yeast vectors; fungi containing fungal vectors; insect cell lines infected with virus (e.g. baculovirus) ; and mammalian cell lines transfected with plasmid or viral expression vectors, or infected with recombinant virus (e.g. vaccinia virus, adenovirus, adeno-associated virus, retrovirus, etc.).

Using methods known in the art of molecular biology, including methods described above, various promoters and enhancers can be incorporated into the vector or the nucleic acid molecule encoding the recombinant protease, to increase the expression of the recombinant protein(s), provided that this increased expression is compatible with (for example, non-toxic to) the particular host cell system used. The selection of the promoter will depend on the expression system used. Promoters vary in strength, i.e. ability to facilitate transcription. Generally, for the purpose of expressing a cloned gene, it is desirable to use a strong promoter in order to obtain a high level of transcription of the gene or the variant sequence and expression into the recombinant protein. For example, bacterial, phage, or plasmid promoters known in the art from which a high level of transcription has been observed in a host cell system comprising E. coli include the lac promoter, trp promoter, tac promoter, reca promoter, ribosomal RNA promoter, the P_R and P_L promoters, lacUV5, ompf, bla, lpp, and the like, may be used to provide transcription of the inserted DNA sequence encoding the recombinant protein.

As known to those skilled in the art, such vectors for expression in mammalian cells can be selected from plasmids, viruses, and retroviruses. For a recent review of vectors useful in gene therapy, see Weichselbaum and Kufe (1997, Lancet, 349:S1O-S12). The features of a vector which make it useful in the methods of the present invention include that it have a selection marker for identifying vector which has inserted therein the nucleotide sequence to be expressed; restriction sites to facilitate cloning; and the ability to enter and/or replicate in mammalian cells. Examples of a preferred vector for the in vivo introduction of a recombinant vector into mammalian cells include, but are not limited to viral vectors. Virus-based vectors are one preferred vehicle as they infect cells in vivo, wherein during the infection process the viral genetic material is transferred into the cells. A retroviral vector, such as a plasmid containing AAV (Adeno-associated virus) sequences, has been described previously (see for

example Chatterjee et al., 1992, Science, 258:1485-1488; U.S. Patent No. 5,252,479, herein incorporated by reference). Examples of other vectors for the in vitro or in vivo introduction into mammalian cells include retroviral vectors (Miller et al., 1989, BioTechriiq-ues 7:980-990; Korman et al., 1987, Proc. Nati. Acad. Sci. USA 84:2150-54), papovavirus episomes (U.S. Patent No. 5,624,820, herein incorporated by reference), and adenovirus vectors (U.S. Patent No. 5,585,362, herein incorporated by reference). Promoters are known to those skilled in the art, and may include viral or viral-like basal promoters like the 5V40 late promoter, the RSV promoter, the CMV immediate early promoter, and a VL3O promoter; and cellular promoters (See, e.g., Larsen et al., 1995, Nucleic Acids Res. 23:1223-1230; Donis et al., 1993, BioTechniques 15:786-787; Donda et al., 1993, Mol. Cell. Endocrinol. 90:R23-26; and Huper et al., 1992, In Vitro Cell Dev. Biol. 28A:730-734).

In one illustration of this embodiment, a nucleotide sequence comprising clone 1 (SEQ ID NO:l) was placed under the control of a tetracycline-regulated promoter in a commercially available plasmid (pTet-Splice; GIBCO). The construction was accomplished in two steps. An amplified product was generated that corresponded to the 3' end of clone 1 cDNA (nucleotide 2391 to nucleotide 2780 of SEQ ID NO:1) using a 5' primer containing an EcoRI restriction site (SEQ ID NO:25) and a 3' primer containing a SpeI restriction site (SEQ ID NO:26). The cycling protocol included 93°C for 35 seconds, 55°C for 60 seconds, 72°C for 3 minutes for 40 cycles followed by a 10 minute incubation at 72°C using a thermostable DNA polymerase. The product was cleaved with EcoRI and SpeI, then cloned into corresponding restriction sites in the plasmid. The resultant plasmid was selected and then sequenced to confirm absence of mutations. This recombinant plasmid was then cleaved with EcoRI and BglII. To reconstitute the open reading frame encoding Lu-ECAM-l, the 2.3 kb EcoRI/Bg1II fragment was excised from clone 3 and inserted into the plasmid. The resulting plasmid, pTet-Splice-Lu-ECAM-1, was then co-transfected into HEK293 cells with another plasmid (pTet-tTAK) that encodes a transcriptional activator specific for the pTet-Splice vector. Transfection was done using a transfection reagent (lipofectamine) according to the manufacturers instructions. Cells were harvested 24 hours after the start of transfection. Immunoblot analysis of the cells using polyclonal R41 resulted in the detection of recombinant Lu-ECAM-l precursor of 120 kDa, and recombinant Lu-

ECAM-l-associated protein of 38 kDa. When the cells were probed in immunoblot with anti-peptide antibody CU8, detected was recombinant Lu-ECAM-1 precursor of 120 kDa, and recombinant Lu-ECAM-l of 90 kDa.

In another embodiment of the invention, mCLCA1 cDNA was cut from the pBluescript vector (Stratagene) with SacI and PvuI, blunt ended with Klenow Polymerase and inserted into the tetracycline sensitive mammalian expression vector (pTet-splice, Life Technologies, Inc.) at the EcoRV site. HEK293 cells were cotransfected with mCLCAl cDNA cloned into the pTet-splice along with a vector expressing a tetracycline activator (pTet-tTak) using standard transfection techniques well known to those skilled in the art and as described above (Lipofectamine, Life Technologies, Inc.). Cells were cotransfected with a reporter vector as described above. In another illustration of this embodiment, human CLCA1, HEK293 cells were transfected with either pcDNA 3.1 containing the CLCA1 insert and a reporter vector (enhanced green fluorescent protein, EGFP, CLONTECH) or the reporter vector alone. Transfection can be carried out by standard techniques known to those skilled in the art including CaPO4 precipitation or Lipofectamine (Life Technologies).

For human CLCA2, HEK293 cells were transfected using Lipofectamine using manufacturer's instructions. For example, 5 ul lipid and 0.5 ul of CLCA2 were cloned into pcDNA 3.1 per 35 mm well in a 2-3 hour incubation. For expression studies, the 2,832 bp CLCA2 ORF was PCR amplified from human trachea poly-A^+ RNA (Clontech) following reverse transcription with Superscript RNase H reverse transcriptase (Life Technologies) and random hexamer priming. PCR was performed with Pwo DNA Polymerase (Boehringer; initial denaturation at 94°C for 3 min, 35 cycles of 94° for 50 s, 58°C for 30s, and 72°C for 2 min with a time increment of 3 s per cycle for each extension step (72°C), followed by a final extension step of 72°C for 8 min). Primer sequences were (upstream primer: SEQ ID NO:41, downstream primer: SEQ ID NO:42 with NotI-linkers underlined). PCR products were gel purified, incubated with NotI, and cloned into the expression vector pcDNA3.1 (Invitrogen). Four different PCR products were sequenced to control for potential PCR-induced sequence errors. Cells were simultaneously cotransfected with a reporter vector as described above. Chloride channel conductance activity was recorded after allowing the cells to recover for 24 hours.

The 2817 bp fragment of the hCLCA3 cDNA cloned into pcDNA3.1 was simultaneously transcribed and translated as described for the other CACC-AMs. Samples were analyzed by 10% SDS-PAGE (5 µl of a 25 µl reaction), followed by drying of the gel and exposure to film for 8 h. Protease protection assays were performed as described to ascertain whether hCLCA3 translation products were translocated into the microsomes and thus entered the secretory pathway. In the presence of microsomal membranes in vitro translated and ^{35S}-labeled wild type hCLCA3 was digested with Proteinase K (Sigma; 100 µg/ml) for 60 min on ice with or without detergent present (0.5% Nonidet-P 40). The reaction was stopped by adding phenylmethylsulfonyl fluoride and the products were analyzed by 10% SDS-PAGE and exposure to film. To allow for immunological detection of the translation products, three immunotagged cDNA clones were constructed (m1 to m3) by inserting a partial sequence of the human c-myc protein (EQKLISEEDL; SEQ ID NO: 47) into the amino termini of the first (m1), the second (m2), or both (m3) ORFS. Generation of these constructs using overlap extension PCR and Pwo DNA polymerase (Boehringer) was as described. Correct sequences of the constructs were verifiedby sequencing. Immunotagged DNA constructs were either in vitro translated as described above or transfected into 70% confluent human embryonic kidney (HEK) 293 or chinese hamster ovary (CHO) cells via the Lipofectamine Plus method (Life Technologies) Cell lysates were harvested after 48 h, resolved via 10% SDS-PAGE, and electroblotted onto nitrocellulose. Blots were probed with mouse-anti-human c-myc antibody 9E10 (1 µg/ml; Calbiochem) as primary antibody, horseradish peroxidase-conjugated goat anti-mouse antibody (0.2 µg/ml) as secondary antibody, and developed using enhanced chemiluminescence (Amersham). Secretion of the recombinant hCLCA3 protein into the culture supernatant was assayed by concentrating the conditioned medium (24 to 48 h after transfection) of HEK 293 or CHO cells transfected with construct m3 using ultrafiltration devices with a molecular cutoff at 10 kDa (Ultrafree- 15, Biomax-l0 filter; Millipore; centrifugation at 2,000 g for 30 min at 4°C).

EXAMPLE 6

This embodiment demonstrates that the CACC-AMs of the present invention can function as adhesion molecules. As an illustration, a recombinant LU-ECAM-1, encoded by a nucleic acid molecule according to the present invention, has unexpectedly improved biological activity. Recombinant (r) Lu-ECAM-l and wild type (wt) Lu-ECAM-l were compared in their adhesion ability to lung-metastatic B16-Fl0 melanoma cells. Using anti-Lu-ECAM-l mAb 6D3, wtLu-ECAM-1 was purified from extracts of bovine aortic endothelial cells, and rLu-ECAM-l was purified from extracts of transfected HEK293 cells. The tumor cell adhesion assay was performed as described previously (Zhu et al., 1992, supra). Briefly, 100 μg/ml in phosphate buffered saline of either wtLu-ECAM-1 or rLu-ECAM-l was used to coat wells of 96 plates overnight at 4°C. Wells were then washed with tissue culture medium, and each well is seeded with a suspension of tissue culture medium and 2 x 10^4 tumor cells which had been radio-labelled. After being spun onto the coated wells at 15 g for 1 minute, and incubated for 10 minutes at 37°C, nonadherent tumor cells were spun off at 150 g for 5 minutes. Adherent tumor cells were then dissolved in 1% SDS and counted in a liquid scintillation counter. Tumor cell attachment is recorded as the percent cells bound of the total cells seeded. Inhibition of tumor cell adhesion is determined by first incubating the Lu-ECAM-l coated wells with mAb 6D3 (l0 μg/ml) for 1 hour at room temperature before the tumor cells are added.

As shown in FIG. 5, recombinant Lu-ECAM-1 has unexpectedly improved biological activity (e.g., adhesive function to lung-metastatic tumor cells) as compared to wild type Lu-ECAM-1. More particularly, rLu-ECAM-l supported adhesion of 87% of lung-metastatic tumor cells, whereas wtLu-ECAM-l supported adhesion of only 43% of lung-metastatic tumor cells. Lung-metastatic tumor cell adhesion to wtLu-ECAM-1 was almost completely blocked by anti-Lu-ECAM-l mAb 6D3, whereas lung-metastatic tumor cell adhesion to rLu-ECAM-1 was only partially inhibited (66%) by the concentration of anti-Lu-ECAM-1 mAb 6D3 used.

EXAMPLE 7

A comparison of the amino acid sequence of the CACC-AMs of the present invention is shown in Figure 9. Sequence alignment and homology searches were

carried out by using standard commercial software. For example, BLAST program was used for homology searches in existing data bases, and Megalign of the DNAStar package (Lasergene) was used for multiple sequence alignment. The sequence alignment of the four CACC-AMs of the present invention and the bovine CLCA (Cunningham et al. supra) indicates conservation throughout the entire length of the sequence, without the compartmentalization of more conserved domains. No significant homologies to any other chloride channel proteins were detected.

Table 1 illustrates a comparison of the size of the various mammalian Lu-ECAM-l proteins and Lu-ECAM-1 associated proteins as encoded-by the respective open reading frames.

Table 1

Species	SEQ ID NO:	Total # of Amino Acids	Predicted Size
bovine	2 and 3	905 a.a.	90 kD, 32-28 kD
human hCLCA1	28	914 a.a.	90 kD, 40 kD
human hCLCA3	30	1000 a.a.	130 kD (processing not known)
human hCLCA2	32	943 a.a.	130 kD **90 kD, 35 kDa**
murine mCLCA	34	902 a.a.	130 kD, 125 kD 90 kD, 32-38 kD

Table 2 is a comparison among the mammalian Lu-ECAM-1 family showing both an approximated amino acid similarity and an approximated amino acid identity (expressed as "similarity/identity").

Table 2

	bovine (SEQ ID	murine (SEQ ID	human (SEQ ID	human (SEQ ID	human (SEQ ID

	NOs:2&3)	NO:34	NO:28)	NO:30)	NO:32)
bovine (SEQ ID NOs:2&3)	100/100	81.3/70.8	67.4/52.4	85.7/77.4	63.7/49.8
murine mCLCA (SEQ ID NO:34)	--	100/100	67.5/52.7	80.9/69.5	62.8/48.4
human hCLCA1 (SEQ ID NO:28	--	--	100/100	65.3/51.4	62.3/44.7
human hCLCA3 (SEQ ID NO:30)	--	--	--	100/100	62.1/48.2
human hCLCA2 (SEQ ID NO:32)	--	--	--	--	100/100

Table 3 is a comparison among the mammalian Lu—ECAM-1 gene family showing approximated nucleic acid similarities (expressed in %).

Table 3

	bovine (SEQ ID NO:1)	murine (SEQ ID NO:33)	human (SEQ ID NO:27)	human (SED ID NO:29)	human (SEQ ID NO:31)
bovine (SEQ ID NO:1)	100	76.7	63.1	85.9	64.4
murine (SEQ ID NO:33)	--	100	62.6	76.1	61.2

human (SEQ ID NO:27)	--	--	100	63.3	58.9
human (SEQ ID NO:29)	--	--	--	100	62.6
human (SEQ ID NO:31)	--	--	--	--	100

EXAMPLE 8

This embodiment illustrates that the full length cDNAs of the present invention encode calcium sensitive chloride channels. The various cDNAs were used for transfection of a cell line. For electrophysiological studies, cells were also cotransfected with a reporter vector (pEGFP, CLONTECH). Cotransfection with a reporter vector allows for easy identification of transfected cells by visualization under a fluorescent microscope. Whole cell recording was then carried out in the transfected cells to determine the presence of calcium sensitive chloride channels.

Transfected cells were used for electrophysiological recording. Cells were superfused with a bath solution containing 112 mM NMDG-Cl, 30 mM sucrose, 1 mM EGTA, 0.366 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM N-2-hydroxy-xyethanylpiperazine-N-2-ethanesulfonic acid. Whole cell channel activity was recorded in transfected cells by using borosilicate glass electrodes (tip resistance 4-9 M ohms) filled with the bath solution. Recordings were carried out in the presence or absence of a calcium channel inhibitors (DIDS, niflumic acid and DTT). To determine the effect of ionomycin on channel activity, electrodes filled with standard bath solution containing either 5 mM ATP and 1 mM EGTA in the presence of low intracellular calcium. After gigaohm seal formation, cells were clamped at +20 mV. Whole cell currents were recorded at room temperature, sampled at 5-10 kHz and filtered at 1-2 kHz. The I-V relationship was determined using 300 ms voltage steps from a holding potential of +20 mV to potentials from -100 to +100 mV at 10 mV intervals. To normalize measured membrane currents to membrane currents to membrane capacitance, the capacitive current transient

recorded in response to a 10 mV hyperpolarizing pulse was integrated and divided by the given voltage to give total membrane capacitance (C_m) for each cell.

As shown in Figure 9, expression of mCLCA1 in HEK293 cells was associated with the appearance of a novel $Ca2^+$ sensitive Cl- channel as determined by whole cell recordings in the presence and absence of the $Ca2^+$ ionophore ionomycin (2 uM). As shown in Figure 9b, at low intracellular free Ca2+ concentrations, the basal current at +100 mV in mCLCA1-transfected cells was 2.05± 1.09 pA/pF. With ionomycin the current increased to 10.23 ± 3.46 pA/pF. No significant effect of these manipulations was seen in non-transfected or control-transfected cells. Basal currents in the presence of 2 mM Ca2+ in transfected cells averaged 12.01 ± 6.31 pA/pF. Perfusion of 300 uM DIDS reduced the current to 1.84
± 0.96. A similar effect was seen with NFA and DTT. These results indicate that the expression of mCLCA1 in HEK293 cells is associated with the appearance of a Ca2+ sensitive chloride conductance. Under whole cell conditions, the current was outwardly rectified and inhibited by the anion channel blockers DIDS and NFA as well as the reducing agent DTT. This data is summarized in Figure 10.

Whole cell recording of cells transfected with hCLCA1 cDNA demonstrated the induction of calcium sensitive chloride channels(Figure 11). External perfusion of ionomycin (2 uM) was associated with an increase in the maximally activated current at +100 mV from 0.65 to 11.06 pA/pF. The current voltage relationship was outwardly rectified and reversed at 0 mV under symmetrical recording conditions. No effect of ionomycin was observed on non-transfected cells or control transfected cells. Addition of DIDS, DTT or niflumic acid reduced the currents to 1.63, 1.67 and 2.07 pA/pF respectively Cell attached patch recordings of single channels confirmed the presence of calcium sensitive anion channel (data not shown). This data is summarized in Figure 12.

Whole cell recordings of hCLCA2 transfected HEK293 cells exhibited s slightly outwardly rectifying current/voltage relationship that was absent from control cells (transfected with vector alone; Figure 13). This current was sensitive to DIDS (300 uM), DTT (2 mM), niflumic acid (100 uM), and tamoxifen (10 uM). When the pipet solution contained low Ca2= (about 25 nM) with 2 mM Ca2+ in the bath, perfusion of the Ca2+ ionophore ionomycin (4 uM) through the bath also activated the current (Figure 13e).

These results indicate that the expression of CACC/AM molecules disclosed herein and their variants is associated with the appearance of calcium sensitive chloride channels.

EXAMPLE 9

This embodiment illustrates uses of the sequences according to the present invention. In one embodiment of the present invention, an individual having a primary tumor having lung-metastatic capabilities is treated with an anti-adhesion therapy comprising administering to the individual a therapeutically effective amount of a composition comprising either antibody raised to rLu-ECAM-l or recombinant Lu-ECAM-1 complex, or a vector for expressing a soluble form of rLu-ECAM-l or rLu-ECAM-l complex which can then bind to the lung-metastatic tumor cells. Either composition may function to prevent lung- metastatic tumor cell adhesion to the lung venule endothelial cells, thereby preventing colonization by the metastatic tumor cells. As known to those skilled in the art, an effective amount of a therapeutic composition may depend on the route of administration (e.g., intravenous or other route known in the art), and physiological factors including the age, size, and rate of metabolism of the individual to be treated.

Another embodiment of the present invention is a method for providing calcium-dependent chloride conductance channels to mammalian cells. Recombinant Lu-ECAM-l or rLu-ECAM-1 complex may form a chloride channel which may affect chloride secretion, and hence fluid secretion, from the cell. It may be that the chloride ion channel is coupled to the adhesion process involving the binding of Lu-ECAM-l to a ligand, as similarly observed for the adherence and growth of lymphatic endothelial cells (Martin et al., 1996, supra). Thus, in mammalian cells in which the membrane chloride ion channels are deficient in number or function (e.g., in airway epithelial cells of cystic fibrosis patients), a method of providing to mammalian cells a calcium-dependent chloride conductance channel, rLu-ECAM-l or rLu-ECAM-l complex, comprises administering directly to the lung endothelial and/or epithelial cells (in vitro or in vivo) an expression vector. The expression vector contains a nucleic acid molecule(or a variant thereof) operably linked to expression control sequences, wherein

the nucleic acid molecule encodes either rLu-ECAM-l or rLu-ECAM-1 complex, with the resultant expression vector being introduced into the mammalian cell, and a functional calcium-dependent chloride conductance channel produced in the mammalian cells which contain the expression vector. The cells targeted for chloride conductance channel production may include airway cells selected from the group consisting of tracheal, bronchial or lung cells. If the cells are transfected in vitro, the transfected cells may then be introduced in vivo into the area of the lungs of the individual which is deficient in chloride channel function.

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EXAMPLE 10

This Example describes the antibodies used where noted in the Examples that follow Example 10. Antibodies against the β_4 integrin ectodomain were mouse α-human monoclonal antibody (mAb) 3E1 (from Dr. E. Engvall, The Burnham Institute, La Jolla, CA), rabbit α-human polyclonal antibody (pAb) H-101 (Santa Cruz Biotechnology, Santa Cruz, CA), and rat α-mouse mAb346–11A (BD Pharmingen), and against the β_4 cytoplasmic domain rabbit α-human pAb1922 (Chemicon, Temecula, CA). Mouse α-human β_1 integrin mAb (clone 18) was from BD Pharmingen, and α-human β_3 mAb25E11 was from Chemicon. Mouse mAb9E10 was against the Myc protein tag (Calbiochem), mouse mAb(F-7) was against the HA tag (Santa Cruz Biotechnology), and rabbit pAb(B-14) and mouse mAb(Z-5) were against glutathione *S*-transferase (GST) (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat α-mouse, α-rat, and α-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-bCLCA2 (Lu-ECAM-1) mAb6D3 was produced in BALB/c mice as previously described (Zhu et al. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9568-9572; Zhuet al (1991) J. Histochem. Cytochem. 39, 1137-1142) and selected for blocking the adhesion of lung-metastatic cancer cells (*e.g.* R3230AC-MET; and B16-F10) to bCLCA2-expressing bovine aortic endothelial cells. The antibody cross-reacts with mCLCA1. Rat plasma fibronectin was from Invitrogen. Purified β_1 (α_5 β_1) and β_3 (α_v β_3) integrins were purchased from Chemicon. Human placental and EHS laminins as well as all other reagents were from

Sigma. Genemed Synthesis, Inc. (South San Francisco, CA) prepared synthetic peptides of β_4(184–203) and β_1(207–213).

EXAMPLE 11

This Example describes the construction of plasmids containing GST-bCLCA2 fragments and plasmids containing integrin fragments. To generate GST fusion proteins from bCLCA2 fragments that together span the length of the 90-kDa bCLCA2 proteins, bCLCA2 cDNA was cut with unique restriction enzymes: (i) GST-HX, HindIII and XhoI; (ii) GST-HV, HindIII and PvuII; (iii) GST-HP, HindIII and PstI; (iv) GST-NE, NdeI and EcoRV; (v) GST-VX, PvuII and XhoI; (vi) GST-PX, PstI and XhoI; and (vii) GST-BX, BstXI and XhoI. Blunt-ended restriction fragments were electrophoretically purified and ligated at either SmaI or blunted EcoRI sites to linearized pGEX-2T vector (Amersham Biosciences).

To construct the GST- $\beta_4BM_{hCLCA2(90)}$ **(SEQ ID NO:48)** (harboring the β_4-binding Motif of hCLCA2), PROTOMAT was used to search for conserved motifs in the 90- and 35-kDa subunits of hCLCA2. Identified sequences AFSRISSGTG, **(SEQ ID NO:50)** located at amino acids 479–488 of the 90-kDa hCLCA2 subunit ($\beta_4BM_{hCLCA2(90)}$), and GFSRVSSGGS **(SEQ ID NO:51)**, located at amino acids 730–739 of the 35-kDa hCLCA2 subunit ($\beta_4BM_{hCLCA2(35)}$; **SEQ ID NO:49)** both tagged at their C termini with hemagglutinin (HA), were generated by primer extension with *Taq* polymerase and inserted into the EcoRI and HindIII sites of pGEX-KG. ($\beta_4BM_{hCLCA2(35)}$ is **SEQ ID NO:49).**

The specific determining loop (SDL) sequences of the β_4 integrin subunit (amino acids 184–203) and the β_1 integrin subunit (amino acids 197–219), tagged at the C terminus with HA, were generated by PCR and inserted into the EcoRI and HindIII sites of pGEX-KG to generate β_4(184–203) and β_1(197–219) GST fusion constructs, respectively.

To generate $\beta_{(4\text{-}1\text{-}4)}$ chimeric integrin, amino acids 184–203 of the SDL of the β_4 I-domain were substituted for the corresponding sequence of the β_1 integrin (amino acids 197–219) by PCR using the unique restriction sites NdeI in the RcCMV vector backbone and BspMI in the β_4 cDNA with the high fidelity DNA polymerase Herculase (Stratagene, La Jolla, CA). The sequence of the chimeric integrin was verified.

EXAMPLE 12

This example describes the cell lines and transfection procedures used in Examples below. The MDA-MB-231L breast cancer cell line was from Dr. J. A. Price (The University of Texas M. D. Anderson Cancer Center, Houston, TX), 4T1 was from Dr. F. R. Miller (Karmanlos Cancer Institute, Detroit, MI), and human embryo kidney (HEK) 293 cells were from ATCC (Manassas, VA). All cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. HEK293 cells were transiently transfected with Myc-tagged hCLCA2, α_6, $\alpha_6 + \beta_4$, and $\alpha_6 + \beta_{4\text{-}1\text{-}4}$, or vector alone using LipofectAMINE™ Plus as described by the manufacturer (Invitrogen). Transfection rates assessed by green fluorescent protein co-transfection were 40–50%. Cells were used in the various assays 48 h after transfection unless otherwise stated.

EXAMPLE 13

This Example demonstrates the purification of GST fusion proteins. GST-bCLCA2 fragments, GST- β_4(184–203)-HA, GST- β_1(197–219)-HA, GST-$\beta_4BM_{hCLCA2(90)}$-HA, and GST-$\beta_4BM_{hCLCA2(35)}$-HA fusion proteins were purified according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Briefly, 2 liters of *Escherichia coli* culture were centrifuged after a 2-h isopropyl-β-D-thiogalactoside induction (0.3 mM). Cell pellets were sonicated in 100 ml of column buffer (CB; 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) and then centrifuged at 19,000 rpm (at 4 °C for 20 min). Supernatants were diluted 1:3 with CB and passed through a glutathione-agarose column. Columns were washed with 10 volumes of CB and eluted with 10 mM glutathione in CB. The purity of the elutes was evaluated by Coomassie Blue staining of SDS-polyacrylamide gels and Western blotting with α-GST and/or α-HA pAbs. Protein concentrations were measured by the Bradford method (Bio-Rad). Alternatively, HA-tagged GST fusion proteins were purified with α-HA mAb-conjugated protein G-agarose beads. Control GST fusion proteins were P14 and PEDA derived from fibronectin (FN) III (14) (amino acids 2045–2062) and $FNIII_{EDA}$ (amino acids 1774–1791), respectively using procedures well known to those skilled in the art.

EXAMPLE 14

This Example demonstrates the purification of hCLCA2 and β_4 integrin. Myc-tagged hCLCA2 was immunopurified from transfected HEK393 cells 48 h after transfection, and the β_4 integrin was immunopurified from MDA-MB-231L cells as described previously using techniques well known to those skilled in the art. Cells were lysed in Tris-buffered saline (TBS) lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.01% aprotinin, 1 mM benzamidine, and 1% octyl-β-glucoside (OG)) (for 1 h at 4 °C), and lysates were centrifuged at 15,000 rpm (for 20 min at 4 °C) to remove insoluble materials. Precleared supernatants were mixed with α-Myc mAb9E10 (hCLCA2) or α-β_4 mAb3E1, respectively, and incubated for 4 h at 4 °C. Protein G-Sepharose beads were then added to the reaction mixtures and incubated overnight at 4 °C. Immune complexes were washed extensively with cold TBS lysis buffer (0.5% OG), and bound protein (hCLCA2 and β_4 integrin) was collected in 100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 100 mM glycine, and 0.5% OG. The purity was determined by SDS-PAGE followed by silver staining and/or Western blotting with α-Myc mAB9E10 or rabbit α-β_4 pAbH101, respectively (4, 10). The 35-kDa subunit of hCLCA2 was purified from extracts of HEK293 cells that had been transfected with a double tagged hCLCA2 cDNA construct containing a Myc tag at its N terminus and an HA tag at its C terminus. The 35-kDa protein was purified by α-HA immunoaffinity chromatography, while the 90-kDa hCLCA2 protein was purified by α-Myc immunoaffinity chromatography from the flow-through of the α-HA column.

EXAMPLE 15

This Example discloses methods for performing ELISA assays in the Examples below. An ELISA was used according to standard methods to measure the binding of GST-$\beta_4BM_{hCLCA2(90)}$-HA to the β integrins β_4, β_1, and β_3. Wells of microtitration plates were coated with integrins, the control substrate fibronectin, or the blocking agent BSA (all at 10 μg/ml) overnight at 4 °C. After blocking with 3% skim milk (for 2 h at room temperature) and several washes with phosphate-buffered saline, GST-$\beta_4BM_{hCLCA2(90)}$-HA was added at various concentrations and incubated for 1 h at room temperature. Bound

GST-$\beta_4BM_{hCLCA2(90)}$-HA was determined colorimetrically using α-HA mAbF-7, horseradish peroxidase-conjugated goat α-mouse IgG antibody, and the horseradish peroxidase substrate *o*-phenylenediamine. For ELISA binding studies between GST-$\beta_4BM_{hCLCA2(90)}$-HA and GST-β_4-HA, GST-β_4-HA was biotinylated, and GST-$\beta_4BM_{hCLCA2(90)}$-HA-bound GST-β_4-HA was detected by streptavidin-horseradish peroxidase as described previously (37).

EXAMPLE 16

This Example discloses the methods of performing pull-down assays. Pull-down assays were performed according to standard methods known to those skilled in the art (i.e., Puzon-McLaughlin, W., and Takada, Y. (1996) J. Biol. Chem. 271, 20438-20443). In brief, immunopurified full-length β_4 integrin, GST-β_4(184–203)-HA, and GST-β_1(197–219) were immobilized on protein G-Sepharose beads conjugated with α-β_4 pAb1922 or glutathione-conjugated agarose beads, respectively. Beads with bound β_4 or GST fusion proteins were washed extensively with lysis buffer containing 1 mM $MnCl_2$ and 0.5% OG (washing buffer) and then incubated overnight at 4 °C with cell lysates or immunopurified hCLCA2 from Myc-hCLCA2-transfected HEK293 cells, both in TBS lysis buffer containing 1 mM $MnCl_2$ at a final detergent concentration of 0.5% OG (4, 40). Conversely, beads conjugated with α-Myc mAb9E10 and bound hCLCA2-Myc or glutathione-conjugated agarose beads with bound GST-$\beta_4BM_{hCLCA2(90)}$-HA or GST-$\beta_4BM_{hCLCA2(35)}$-HA were used to pull down theβ_4 integrin from lysates of HEK293 cells co-transfected with the α_6 and β_4 integrin subunits or surface-biotinylated MDA-MB-231 cells (both cell lysates were prepared in the same 0.5% OG-containing buffer as above). For detection of bound protein, beads were washed extensively with washing buffer and boiled in SDS sample buffer, and bound material was detected by SDS-PAGE and Western blotting.

EXAMPLE 17

This Example demonstrates fluorescence activated cell sorting (FACS) analyses and adhesion and lung colony assays. FACS analyses, adhesion assays, and lung colony assays were performed according to procedures well known to those skilled in the art. (See, i.e., Abdel-Ghany, et al. (2001) J. Biol. Chem. 276, 25438-25446; Cheng et al.

(2003) J. Biol. Chem. 278, 24600-24607.) Briefly, tumor cell binding of GST-CLCA$_{(90)}$-β_4BM was determined by incubating MDA-MB-231 cells (or 4T1) in end-over-end culture with GST-β_4BM$_{hCLCA2(90)}$-HA for 20 min at room temperature in Dulbecco's modified Eagle's medium containing 1% BSA. After washing, tumor cells were stained with α-HA mAb (or mIgG as control) and subjected to FACS analysis. β_4/CLCA adhesion inhibition experiments were conducted with both hCLCA2- and β_4-derived polypeptides. Human CLCA2-derived polypeptides (GST-β_4BM$_{hCLCA2(90)}$-HA and GST-β_4BM$_{hCLCA2(35)}$-HA) or control polypeptides (GST-P14-HA and GST-PEDA-HA) were preincubated with tumor cells for 20 min, and tumor cell adhesion to hCLCA2-coated dishes was performed in the presence or absence of polypeptide. β_4-derived polypeptides (β_4(184–203) (synthetic), GST-β_4(184–203)-HA, β_1(197–219) (synthetic), and GST-β_1(197–219)-HA (controls)) were incubated with hCLCA2-coated dishes for 30 min at 37 °C, and tumor cell adhesion was determined in the presence or absence of polypeptide. Polypeptides were used at the indicated concentrations. Lung colony inhibition assays were performed with hCLCA2-derived polypeptides (GST-β_4BM$_{hCLCA2(90)}$-HA and GST-P14-HA (control) (37)). Tumor cells (1×10^5 cells/mouse) were incubated with hCLCA2 polypeptide (for 20 min at 37 °C) prior to intravenous injection together with polypeptide (0.5 mg/mouse). Female Scid/beige (MDA-MB-231) and BALB/c (4T1) 4-week-old mice were used (eight mice/experimental condition).

EXAMPLE 18

This Example demonstrates that CLCA proteins contain binding domains for β_4-expressing tumor cells. To identify the CLCA sequence that is responsible for the β_4/CLCA-mediated adhesion of lung-metastatic human (MDA-MB-231) and mouse (4T1) breast cancer cells, we first examined the binding behavior of the β_4/CLCA adhesion-blocking mAb6D3 using a series of polypeptides encompassing the length of the 90-kDa subunit of the CLCA prototype bCLCA2 (Lu-ECAM-1) (Fig. 15A). Polypeptides were prepared as GST fusion proteins in *E. coli* as described in Example 11, and the fusion proteins were purified as described in Example 13 on a glutathione column (Fig. 15B). Antibody 6D3 was able to bind and immunoprecipitate the fusion proteins GST-HX, GST-VX, GST-PX, and GST-BX but not GST-HV, GST-HP, and GST-NE (Fig. 15C). The

shortest bCLCA2 fragment recognized by mAb6D3 was GST-BX, localizing the antibody-binding domain to the second extracellular domain of bCLCA2 (Elble et al. (1997) J. Biol. Chem. 272, 27853-27861). Next we examined whether the same bCLCA2 fragments that supported binding of the β_4/CLCA adhesion-blocking antibody also harbor the binding domain for β_4-expressing MDA-MB-231 cells. To do so, we coated wells of microtitration plates with GST-BX, GST-PX, GST-NE, and GST-HP and seeded coated wells with MDA-MB-231 cells. Analogous to the binding characteristics of the adhesion-blocking antibody, MDA-MB-231 bound to GST-PX and GST-BX but not to GST-HP and GST-NE(Fig. 15D). Binding of MDA-MB-231 to GST-PX and GST-BX was equally as strong as the adhesion to the 90-kDa natural processing product of bCLCA2. Unexpectedly, adhesion of MDA-MB-231 as well as mAb6D3 (data not shown) was not restricted to the 90-kDa protein but was also mediated by the 35-kDa subunit of bCLCA2 (Fig. 15D). Curious whether this adhesion behavior was specific for bCLCA2 or extended to other CLCA proteins, we tested the adhesion of the 90- and 35-kDa subunits of hCLCA2 for adhesion to MDA-MB-231 cells. Both products bound the cancer cells (Fig. 15D) but not mAb6D3, which is consistent with its specificity for bCLCA2 and mCLCA1. Identical binding data were also obtained for other lung-metastatic cancer cell lines including 4T1, B16-F10, and CSML-100 using the 90- and 35-kDa subunits of either bCLCA2 (Lu-ECAM-1) or hCLCA2 in static adhesion assays (data not shown).

EXAMPLE 19

The Example demonstrates identification of the β_4 Integrin-binding Motif of CLCAs. We examined whether the 90- and 35-kDa subunits of hCLCA2 (and bCLCA2) harbor a common binding motif for the β_4 integrin by using the PROTOMAT motif search program. The sequences AFSRISSGTG **(SEQ ID NO: 50)** in the 90-kDa and GFSRVSSGGS **(SEQ ID NO: 51)** in the 35-kDa subunits of hCLCA2 were identified as the single, common motif (Fig. 16A). The first sequence is located at amino acid residues 479–488 of hCLCA2, placing it within the GST-BX fragment of bCLCA2 (AFSRISSRSG) **(SEQ ID NO: 50)** recognized in Example 18 as the shortest bCLCA2 fragment to mediate binding of lung-metastatic cancer cells (Fig. 15D). The second sequence is located at amino acids 740–749 of hCLCA2 located near the N terminus of the

35-kDa hCLCA2. To prove that this motif is binding MDA-MB-231 cells via the β_4 integrin, we generated a HA-tagged GST fusion protein of the 90-kDa β_4-binding motif of hCLCA2 (termed $\beta_4BM_{hCLCA2(90)}$) as described in Example 11 and tested its binding ability for the β_4 integrin by ELISA as described in Example 15.

$\beta_4BM_{hCLCA2(90)}$ bound to immobilized β_4 integrin but not to β_1 integrin, β_3 integrin, fibronectin, or BSA. The same result (Fig. 16B) was achieved in the pull-down assays performed as described in Example 16. Immobilized on glutathione-conjugated agarose beads, $\beta_4BM_{hCLCA2(90)}$ pulled down β_4 but not β_1 and β_3 from solutes (Fig. 16C), and, in reverse, β_4 integrin but not β_1 integrin, immobilized by anti-integrin antibodies on protein G-conjugated agarose beads, pulled down soluble $\beta_4BM_{hCLCA2(90)}$ (Fig. 16D). Identical results were obtained with the 35-kDa β_4-binding motif of hCLCA2 (termed $\beta_4BM_{hCLCA2(35)}$) (data not shown). To test whether the adhesion of $\beta_4BM_{hCLCA2(90)}$ to β_4 integrin was dose-dependent, we coated wells of microtitration plates with a standard concentration of immunopurified β_4 integrin (10 μg/ml) and determined the adhesion of increasing concentrations of $\beta_4BM_{hCLCA2(90)}$ by ELISA. Our data showed a linear increase in adhesion of $\beta_4BM_{hCLCA2(90)}$ from 1 to 1,000 ng (Fig. 17A). This adhesion was dependent upon the presence of Mn^{2+}, but not Mg^{2+} or Ca^{2+}, in the assay medium (Fig. 17B).

EXAMPLE 20

This Example demonstrates that $\beta_4BM_{hclca2(90)}$ binds to lung-metastatic cancer cells and inhibits adhesion to cells expressing hCLCA2. To establish hCLCA290- β_4BM as a β_4/hCLCA2 adhesion-blocking polypeptide, we first examined the ability of the polypeptide to bind to the surface of lung-metastatic MDA-MB-231 cancer cells as described in Example 17 and as follows. $\beta_4BM_{hCLCA2(90)}$ was incubated with tumor cells for 20 min at room temperature, and bound polypeptide was detected by α-GST antibody and quantified by FACS analysis. Data showed strong binding of $\beta_4BM_{hCLCA2(90)}$ to tumor cell surfaces, while the control polypeptide P14 did not adhere (Fig.18A, *a*). The FACS histogram generated by bound $\beta_4BM_{hCLCA2(90)}$ was similar to that generated by α- β_4 antibody staining of MDA-MB-231 cells (Fig.18A, *b*), concurring with the interaction between $\beta_4BM_{hCLCA2(90)}$ and the β_4 integrin. In accordance, $\beta_4BM_{hCLCA2(90)}$ as well as

$\beta_4BM_{hCLCA2(35)}$ immobilized on the well bottom of microtitration plates supported adhesion of MDA-MB-231 cancer cells to the same extent as full-length, immunopurified hCLCA2, while BSA and GST did not support tumor cell adhesion (Fig. 18B). Finally $\beta_4BM_{hCLCA2(90)}$ and $\beta_4BM_{hCLCA2(35)}$ were tested for their abilities to block the adhesion of lung-metastatic MDA-MB-231 cells to hCLCA2 -coated wells *in vitro*. Both $\beta_4BM_{hCLCA2(90)}$ and $\beta_4BM_{hCLCA2(35)}$, preincubated with hCLCA2-coated wells for 20 min at room temperature, completely blocked the adhesion of MDA-MB-231 cells to hCLCA2 (Fig. 18C). The control polypeptides P14 and PEDA were unable to block tumor cell adhesion to hCLCA2. Identical results were obtained for lung-metastatic 4T1 murine breast cancer cells (data not shown).

EXAMPLE 21

This Example demonstrates that $\beta_4BM_{hclca2(90)}$ blocks lung colonization of MDA-MB-231 cells in vivo. To examine the effect of the $\beta_4BM_{hCLCA2(90)}$-GST fusion protein on lung colonization of MDA-MB-231 cells, we incubated MDA-MB-231 cells for 20 min with $\beta_4BM_{hCLCA2(90)}$ and then injected tumor cells together with the fusion protein into the lateral tail vein of 4-week-old, female Scid/beige mice (2×10^5 tumor cells/300 μg of $\beta_4BM_{hCLCA2(90)}$/0.2 ml of Dulbecco's modified Eagle's medium/mouse). Control mice received tumor cells preincubated with GST administered at the same dose. Eight weeks later, animals in the control group exhibited signs of respiratory distress, and the experiment was terminated. Autopsy and lung colony counting revealed a median number of >100 (from 77 to >100) tumor colonies in the control group and zero (from 0 to 2) colonies in the $\beta_4BM_{hCLCA2(90)}$-treated animal group (Fig. 19*A*). Gross examination of the lungs showed numerous tumor nodules throughout the lungs as well as in mediastinal and bronchial lymph nodes in GST-treated mice and normal, tumor-free lung in seven of eight $\beta_4BM_{hCLCA2(90)}$-treated mice. Histological examination of the lungs confirmed the gross findings. There was massive tumor involvement in the lungs of GST-treated mice but no evidence of metastatic disease in seven of eight $\beta_4BM_{hCLCA2(90)}$-treated mice. The remaining $\beta_4BM_{hCLCA2(90)}$-treated mouse had two small lung colonies. This outcome was not the result of diminished growth and survival rates of tumor cells exposed to $\beta_4BM_{hCLCA2(90)}$ polypeptide (data not shown).

EXAMPLE 22

This Example demonstrates the determination that the specific determining loop of the β_4 integrin harbors the a domain to which the CLCA β_4 binding domain binds. To identify the β_4 sequence that interacted with hCLCA2, we generated a GST- β_4(184–203)-HA fusion protein (GST- β_4 for short) that corresponded to a predicted loop of the β_1 and β_3 integrins shown to be involved in ligand binding (Fig. 20*A*). This sequence comprises the N-terminal two-thirds of the SDL region of the β_4 integrin subunit. The corresponding sequence of the β_1 integrin was used to prepare a control GST fusion protein (GST-β_1(197–219)-HA (GST- β_1 for short)). These fusion proteins were tested first for their ability to bind hCLCA2 in a modified ELISA. Wells of microtitration plates were coated with GST- β_4, GST- β_1, or GST (all at 10 μg/ml), and coated wells were probed for hCLCA2 adhesion. Human CLCA2 adhesion to GST- β_4-coated wells was more pronounced than the binding of hCLCA2 to high protein-binding plastic, while GST- β_1- and GST-coated wells did not support hCLCA2 binding (Fig. 20*B*). These binding data were confirmed in pull-down assays in which GST- β_4 and GST- β_1 bound to glutathione-agarose beads were tested for their abilities to pull down hCLCA2 from lysates of hCLCA2-Myc-transfected HEK293 cells. Only GST- β_4 but not GST- β_1 was able to pull down hCLCA2 (Fig. 20*C*). To examine whether GST- β_4 and $\beta_4BM_{hCLCA2(90)}$ were the interacting binding domains of the β_4 integrin subunit and hCLCA2, microtitration plates were coated with skim milk (blocking agent), $\beta_4BM_{hCLCA2(90)}$, or the control polypeptide PEDA. Biotinylated GST- β_4 selectively bound to $\beta_4BM_{hCLCA2(90)}$ but not to PEDA (Fig. 20*D*). GST- β_1 did not bind to any of the three substrates (data not shown). Similarly the chimeric β_4 protein $\beta_{4\text{-}1\text{-}4}$ in which the C-terminal two-thirds of the β_4 SDL domain were replaced with the corresponding region of the β_1 integrin subunit failed to bind to hCLCA2 (Fig. 20*E*). Finally synthetic peptides of β_4(184–203) and β_1(207–213) were evaluated for their ability to block the adhesion of MDA-MB-231 and 4T1 breast cancer cells to hCLCA2 and mCLCA1, respectively. Adhesion assays were performed as described in Example 17. (See also, Abdel-Ghany et al., (2001) J. Biol. Chem. 276, 25438-25446; Tsuruta, et al. (2003) J. Biol. Chem. 278, 38707-38714). In brief, wells of microtitration plates were coated with substrate (hCLCA2 (Fig. 21A), mCLCA1 (Fig. 21D), EHS

laminin (Fig. 21C and Fig. 21E), or placental laminin (Fig. 21B)) overnight at 4 °C at the indicated concentration, then seeded with MDA-MB-231 (Fig. 21A, Fig. 21B, and Fig. 21C) or 4T1 (Fig. 21D and Fig. 21E) breast cancer cells, and incubated for 20 min at 37 °C. The number of bound tumor cells was determined by a standard colorimetric method. Polypeptide β_4(184–203) and β_1(197–219) were added to substrate-coated wells and incubated for 30 min at room temperature. Polypeptides were either removed by washing prior to the addition of tumor cells (adhesion blocking) or were present throughout the tumor cell adhesion assay (adhesion competition). A complete inhibition of adhesion (by blocking or competition) was observed for both MDA-MB-231 and 4T1 cells with the β_4 polypeptide but not the β_1 polypeptide. Thus, the β_4 polypeptide, but not the β_1 polypeptide, blocked adhesion of both MDA-MB-231 and 4T1 cells to the respective human and mouse CLCA proteins (Fig. 21, *A* and *D*). Polypeptides were equally efficient in their inhibitory activities when they were preincubated with CLCA-coated wells prior to seeding of tumor cells or when they were present throughout the adhesion assay. Polypeptides had no effect on the binding of tumor cells to placental (Fig. 21*B*) and EHS (Fig. 21, *C* and *E*) laminins.

EXAMPLE 23

This Example demonstrates that the 90-kda protein of hCLCA1 harbors a disrupted β_4BM and fails to bind β_4 integrin. In contrast to the highly conserved β_4BMs of the 90-kDa subunits of hCLCA2, mCLCA5, mCLCA1, and bCLCA2, the hCLCA1 90-kDa hCLCA1 protein exhibited a disrupted β_4BM (see *underlined* deviations from hCLCA2 Fig. 22A). However, hCLCA1 contains a relatively well conserved β_4BM motif (CFSRTSSGGS) **(SEQ ID NO:52)** in its 35-kDa processing product **(SEQ ID NO:53)** (Fig. 22A). Thus, β_4 integrin should not be able to bind to the 90-kDa hCLCA1 protein but might bind to the unprocessed 125-kDa and the processed 35-kDa proteins of hCLCA1. To examine this premise, we tested the binding of the 90-kDa fragment with that of the full-length, unprocessed hCLCA1 prepared from Myc-hCLCA1-transfected HEK293 cells for β_4 binding and MDA-MB-231 adhesion (Fig. 22 *B* and Fig. 22*C*). We transfected HEK293 cells with Myc-tagged hCLCA1 and purified the protein by -Myc immunoaffinity chromatography. Four fractions were collected from the affinity column.

Fractions 1 and 4 contained only the 90-kDa processing product, while fractions 2 and 3 contained the 90-kDa processing product as well as the 125-kDa full-length, unprocessed hCLCA1 (the untagged 35-kDa hCLCA1 was lost in the column flow-through) (Fig. 22B). To test these fractions for adhesion of MDA-MB-231 cells, wells of microtitration plates were coated with the four fractions, then seeded, and incubated for 20 min with MDA-MB-231 cells. Tumor cells strongly bound to fractions 2 and 3 but failed to bind to fractions 1 and 4, indicating that they did not recognize the disrupted "β_4BM" sequence but recognized the sequence of the conserved β_4BM in the 35-kDa fragment of full-length hCLCA1 protein (Fig. 22C). A pull-down assay using GST-4 immobilized on glutathione-agarose beads confirmed these data showing the inability of β_4 to pull down the 90-kDa hCLCA1 but an excellent pull-down of the 90-kDa hCLCA2 (Fig. 22D).

Having described the preferred embodiments of the present invention, it will be apparent to one of ordinary skill in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.